CHOLERA
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## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preface</td>
<td>7</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>9</td>
</tr>
<tr>
<td>Chapter 1. History of the disease</td>
<td>11</td>
</tr>
<tr>
<td>Chapter 2. World incidence, <em>written in collaboration with S. Swaroop</em></td>
<td>51</td>
</tr>
<tr>
<td>Chapter 3. Bacteriology</td>
<td>97</td>
</tr>
<tr>
<td>Chapter 4. Problems in immunology, <em>written in collaboration with W. Burrows</em></td>
<td>202</td>
</tr>
<tr>
<td>Chapter 5. Bacteriophage investigations</td>
<td>373</td>
</tr>
<tr>
<td>Chapter 6. General pathology and morbid anatomy</td>
<td>397</td>
</tr>
<tr>
<td>Chapter 7. Practical laboratory diagnosis</td>
<td>523</td>
</tr>
<tr>
<td>Chapter 8. Clinical pathology</td>
<td>607</td>
</tr>
<tr>
<td>Chapter 9. Symptomatology, diagnosis, prognosis and treatment</td>
<td>684</td>
</tr>
<tr>
<td>Chapter 10. Epidemiology</td>
<td>820</td>
</tr>
<tr>
<td>Chapter 11. Prevention and control</td>
<td>893</td>
</tr>
<tr>
<td>Annex. Examination of cholera-suspect stool specimens, <em>written in collaboration with W. Burrows</em></td>
<td>991</td>
</tr>
<tr>
<td>Index</td>
<td>1001</td>
</tr>
</tbody>
</table>
Preface

It would be no exaggeration to say that it was through cholera, and the fear to which its pandemic sweeps gave rise, that international solidarity in matters of health was born. Cholera was the principal disease covered by the early international sanitary conventions and came at the head of the list of quarantinable diseases. Koch’s discovery of the cholera vibrio, and thus the confirmation of the contagion theory, in 1884 was a scientific keystone of the greatest importance and lay at the base of much progress in the drafting of future sanitary conventions. While in Europe cholera has not been seen since the early twentieth century, its endemic foci in Asia remain, occasionally erupting into epidemics. The disease continues to claim an annual toll of tens of thousands on that continent and to menace other parts of the world, as witnessed by the 1947 epidemic in Egypt whence the disease had disappeared since 1919.

Much has been written on cholera in the past hundred years, but a great part of the work is scattered among the periodicals of the world and is often not easily accessible. Some of this work has proved to be of transitory importance; some, on the other hand, now fallen into neglect, merits inclusion in the history of medical discovery. To-day research workers are still at grips with a number of problems, among them the immunological characteristics of the vibrio and their implications for cholera vaccine; differential bacteriological and biochemical diagnosis and phage typing; and a variety of practical questions of prevention and treatment depending on these.

To assist public health services responsible for cholera control in endemic areas and to provide guidance for those who may one day be faced with the problem in countries now free from the disease, the World Health Organization invited Dr R. Pollitzer to prepare a monograph on cholera. The eleven chapters which constitute the main part of this book originally appeared as separate articles in the Bulletin of the World Health Organization.
over a period of three years; they have now been revised and brought up
to date in the light of comments received and new information made
available during that period.

Dr Pollitzer writes with an authority derived from a long career devoted
largely to the fight against cholera and plague. His monograph on the latter
disease, published by WHO in 1954, is already recognized as a classic;
the World Health Organization publishes the present work in the confident
hope that it will receive the same acclaim.
ACKNOWLEDGEMENTS

During the three years which he devoted to the compilation of the present book, the author has had the benefit of much assistance, advice and encouragement from many institutions and persons. Impossible as it is to enumerate all of them, he wishes nevertheless to record his particularly great indebtedness to the following: The Regents of the University of California most generously accorded to the writer the status of a research associate attached to the George Williams Hooper Foundation, Medical Center, San Francisco. The director of this institution, Dr K. F. Meyer, and also Dr B. Eddie, not merely granted him adequate facilities for writing the text, but were indefatigable in helping the author in every possible way and constantly encouraging him. Most kindly given help in administrative matters was received from Professor H. G. Johnstone, Dean of Students in the Medical Center. Even so it would have been impossible to complete the work had not the author been given most generous grants-in-aid first from the Division of Research Grants and Fellowships of the National Institutes of Health, US Department of Health, Education, and Welfare; then by the Foundation for Microbiology, Rutgers University, New Brunswick, N.J., USA, and finally by the World Health Organization.

The writer's great responsibility in compiling this monograph has been much alleviated by the willingness of two colleagues outstanding in cholera research to participate in his labours: Dr Satya Swaroop, who combines a most authoritative knowledge of medical statistics with a thorough acquaintance with the cholera problem in India, kindly consented to be mainly responsible for the part of the work dealing with the present incidence of the disease; similarly, Dr William Burrows put the author under a deep obligation by participating in the most difficult task of discussing the problems of cholera immunology, to the elucidation of which his own researches have so much contributed. The author is also much indebted to Dr Jean Gallut of the Institut Pasteur, Paris, who has undertaken for the World Health Organization the translation into French of this book, for constantly furnishing information on his own important cholera research work.

It might seem at first glance that San Francisco, now far in time as well as in space from any cholera manifestation, would hardly be a proper locale for making a study of the problems of this disease. Actually, however, the rich main library of the Medical Center in combination with the literary treasures possessed by the Hooper Foundation furnished the author with most of the information which he needed, while certain series of publications, which could not be found on the campus, were fortunately within easy reach in the Lane Library of Stanford University, San Francisco. The author has to thank the staffs of these libraries not only for permission to consult their files, but also for going far out of their way to make these studies particularly easy and enjoyable. On the comparatively rare occasions on which help was required from libraries outside San Francisco, the needs of the author were most obligingly responded to by the Library of the World Health Organization in Geneva or, in a few instances, by the National Library of Medicine (formerly Surgeon General's Library) in Washington, D.C.
Chapter 1

HISTORY OF THE DISEASE

Earliest Evidence

The evidence adduced to prove that epidemic or, as it is commonly called, Asiatic cholera, a specific infection caused by the *Vibrio cholerae*, was present in ancient India has been differently evaluated by different writers. Some of those in favour of an early existence of the disease pointed to descriptions of a syndrome showing clinical features identical with those of true cholera in the ancient Indian medical literature, particularly in the writings of Susruta. However, Macnamara (1876), in his classical *History of Asiatic cholera* pointed out with much reason that

"Hippocrates, Galen and Wang-shooho have left us equally vivid accounts of this form of cholera in the various countries in which they lived... But the more carefully we study the writings of these early authorities, the clearer it appears that they had never met with cholera in its epidemic or Asiatic form".

Sticker (1912), while sharing the misgivings expressed by Macnamara regarding possible references to true cholera in the classical Indian medical works, which were mute in regard to the epidemic prevalence of the choleratic disease they described, drew attention to the following quotation taken by Schmidt (1950) from a Sanskrit work, believed to have been written in Tibet during the reign of Ti-song De-tsen, i.e., during the period from A.D. 802 to A.D. 845:

"When the strength of virtues and merits decreases on earth, there appear amongst the people, first among those living on the shores of big rivers, various ailments which give no time for treatment, but prove fatal immediately after they appeared. At times the *nya* carries away the fourth part of the *dschambudwip* (?), it suddenly destroys the vigour of life and changes the warmth of the body into cold, but sometimes this changes back into heat. The various vessels secrete water so that the body becomes empty. The disease is propagated by contact and infection. The *nya* kills invariably. Its first signs are dizziness, a numb feeling in the head, then most violent purging and vomiting". [Trans.]

While certain that this was a description of true cholera, Sticker expressed doubts regarding the authenticity of the text—a point which it would be of great interest to settle.
However, even if this reference should prove unreliable, there is a second category of evidence which testifies to the early existence of cholera in India by showing that ancient religious rites were invoked to ward off the ravages of this disease.

Macnamara stated in this connexion that the people in Lower Bengal had for a long time past worshipped the goddess of cholera, it appearing, "according to tradition, that, at an early period, the date of which cannot now be ascertained, a female while wandering about in the woods met with a large stone, the symbol of the goddess of cholera. The worship of the deity through this stone was, according to the prevailing ideas of the Hindoos, the only means of preservation from the influence of this terrible disease. The fame of the goddess spread and people flocked from all parts of the country to come and pray at her shrine in Calcutta".

As aptly pointed out by Macpherson (1872), whom Macnamara quoted, the malady must have raged at times with violence, or it would not have been found necessary to propitiate the deity specially on account of it.

Sticker maintained, on the authority of Sanderson (1866) and of Tholozan (1868), that there was in a temple at Gujrat in western India a monolith dating back to the time of Alexander the Great, the inscription of which referred apparently to true cholera, saying:

"The lips blue, the face haggard, the eyes hollow, the stomach sunk in, the limbs contracted and crumpled as if by fire, those are the signs of the great illness which, invoked by a malediction of the priests, comes down to slay the braves..." [Trans.]

While these statements strongly suggest that cholera has existed in India since immemorial times, irrefutable proof of its presence in historical times is furnished by the records of European observers who, after the arrival of Vasco da Gama on the coast of Malabar in A.D. 1498, had been given an opportunity to get acquainted with what was formerly a terra incognita to them. As emphasized by Macnamara,

"it is remarkable that in one of the very earliest communications of this description, written by a European, we have a clear and distinct reference made to Asiatic cholera, and this was the first account of the disease ever published. Doubtless, Asiatic cholera has flourished in the Delta of the Ganges, we know not for how long, but its ravages had not been witnessed by those capable of describing the disease".

This early record, written by Gaspar Correa under the title Lendas da India (i.e., Legends of India) referred to (a) a high mortality observed during the spring of the year 1503 in the army of the sovereign of Calicut, enhanced "by the current spring diseases, and smallpox besides which there was another disease, sudden-like, which struck with pain in the belly, so that a man did not last out eight hours' time"; and (b) an outbreak in the spring of 1543 of a disease called "moryxy" by the local people, the fatality-rate of which was so high that it was difficult to bury the dead. As described by Correa,
“so grievous was the throe, and of so bad a sort that the very worst of poison seemed there to take effect, as proved by vomiting, with drought of water accompanying it, as if the stomach were parched up, and cramps that fixed in the sinews of the joints and of the flat of the foot with pain so extreme that the sufferer seemed at point of death; the eyes dimmed to sense, and the nails of the hands and feet black and arched.”

Since Correa’s time descriptions of cholera manifestations continued to be given by other Portuguese, then by Dutch, French, and British observers, Macpherson in his Annals of cholera quoting 64 records by independent authorities referring to the presence of the disease from 1503 to 1817, ten of whom distinctly mentioned an epidemic spread of the manifestations they described. It was inevitable that these reports were restricted at first to Goa, the only province known to Europeans during the 16th century (Macnamara, 1876). Afterwards, however, other areas on the west coast of India were mentioned successively. Thus Thevenot (1689), who himself contracted the infection, and Fryr (according to Macnamara the first Englishman who wrote about the disease) testified to the presence of cholera on the coast of Surat “some time prior to 1678” (Macnamara). As noted by Sticker (1912), Daman (Damão) near Bombay was affected in 1695.

That the early records referred exclusively to the west coast of India appears to be due not merely to the circumstance that the British gained a foothold on the Coromandel coast and in Bengal in the east more than a century after the Portuguese had reached Goa. Macnamara noted in this connexion that one of the earliest accounts of the occurrence of cholera in India from the pen of an English physician (Dr. Paisley) and dated Madras (on the Coromandel coast), February 1774, was brought to light only 33 years afterwards, when it was printed in Curtis’s work on the Diseases of India... (Edinburgh, 1807)—obviously because most of the early British observers insisted upon classifying the disease among the spasmodic affections instead of recognizing it as an affection sui generis, and designating it Asiatic cholera. Therefore, Macnamara concluded, it was not surprising that no descriptions of this disease were given in the writings of British physicians even during the later part of the eighteenth and at the beginning of the nineteenth centuries. Moreover, as stated by this author, “our possessions in India prior to 1781, were surrounded by large provinces regarding whose habitants we had literally no knowledge whatever; unto those territories the course of the epidemic could not possibly be traced.”

It also deserves attention that the Hospital Board in Madras and Calcutta was established only in the year 1786, so that before that year no regular reports on the incidence of cholera among the Europeans and the native soldiers were available.

Nevertheless, sufficient evidence exists to prove that during the last quarter of the eighteenth century cholera was not only met with on the east
coast as well as in the west of India, but even spread beyond the confines of the sub-continent. However, before dealing with these developments it is necessary to devote attention to the question whether such a spread afield took place during previous times.

General agreement exists that this question must be answered in the negative as far as Europe is concerned, even though a malady clinically identical with true cholera, and often designated by this name, has been described by Hippocrates and many subsequent writers, some of whom used other names for the ailment, e.g., that of weisse Ruhr. It is true that this choleraic disease did not occur solely in sporadic form but that cases of this nature were not infrequently numerous and grouped together, the appearance of this forme catastique of cholera being often ascribed to suitable atmospheric conditions (see, for example, Fabre & Chailan, 1835). However, even though the disease was apt to become prevalent at times, it never showed a truly epidemic spread. This was emphasized by Macnamara, who, referring to the manifestations of what Sydenham called cholera during the period 1679-82 in London, stated that "Sydenham makes no mention of a widely disseminated outbreak of the disease and Wells expressly states that the country was quite free from the malady, and in fact one of its characteristic features was that its ravages were confined to the city of London".

Macnamara concluded, therefore, that the "cholera" manifestations observed by Sydenham and others stood in a relation to the true form of the disease similar to that between the bilious remittent fever of Bengal and the yellow fever of the West Indies. For "the symptoms of a severe attack of bilious remittent fever are very similar to those present in cases of yellow fever; nevertheless we cannot doubt that the two affections are produced by different causes, and that yellow fever is communicable; whereas we are equally sure that bilious remittent fever is due to local influences and is certainly not transmissible by those affected with it to healthy people."

While these and many other observations render it certain that no long-distance spread of cholera from India westwards took place before the nineteenth century, this cannot be so confidently asserted in the case of China to the east.

It is true that, as Wong & Wu Lien-teh (1934) aptly put it, "the term 'huo luan', the present name for cholera, is found in the Nei Ching and other old chronicles, but it appears that it does not refer to the disease we now recognize as Haeser maintained, "the etymology of the term 'cholera' is uncertain. Celsus and others think it derived from χόλη, the bile; Alexander Trallianus from χολίους, the intestines. Kraus ('Kritisch-etymologisches medizinisches Lexicon') and Littre ('Dictionnaire de médecine') are in favour of the derivation from γαλακτός, i.e., the case (gutter). It speaks for this assumption that later Greek writers usually add the word μορBUS (cholera morbus)". However, modern writers seem in favour of the derivation from γαλακτός, Macleod (1910), for instance, declaring that the Hippocratic term cholera originally meant bilious diarrhoea.

It is significant, for instance, that the Arabian medical writers, when confronted by the 1821 cholera outbreak in Oman (see page 19), had no name by which to designate the disease.
as cholera. There is little doubt that in the past this term has been used to cover a group
of affections, such as acute gastro-intestinal infections, colic, appendicitis, ptomaine
poisoning, etc., and cholera might have been mixed up with them. A significant point,
however, is that no one, until at a late period, alludes to the epidemic character of the
disease”.

Nevertheless, Wong & Wu Lien-teh did not believe that true cholera
was entirely absent from ancient China, stating that “one is perhaps
justified in saying that it was present in this country in the 7th century”.

Whether further importations of cholera into China took place before
the nineteenth century seems uncertain. Simmons, in a report published in
1879, stated in this connexion that according to Cleyer, an American author
writing in 1873, the disease, probably, imported from Malacca, appeared
in China in 1669 and also claimed that Le Gentil (1779) in a work entitled
*Voyages dans les mers de l’Inde*... referred to an importation of cholera
into China in the eighteenth century, soon after the disease had been present
on the Coromandel coast in 1761 and 1769. However, while it is possible
that Le Gentil made such a statement in one of his contributions to the
*Mémoires de l’Académie Royale des Sciences*, no reference to the spread of
cholera from India to China could be found in his book, the two volumes
of which appeared in Paris in 1779 and 1781 respectively.

There can be little doubt that, as Cleyer (quoted by Simmons, 1879)
suggested in connexion with Malacca, early importations of cholera took
place from India into neighbouring or not far distant countries, particu­
larly into Burma. However, the only seventeenth century reference available
in this respect deals with an appearance of the disease in Batavia, Java, in
1629 observed by Bontius, surgeon to the Dutch East India Company,
who recorded that the Governor-General succumbed to the infection
(Macnamara, 1876; Proust, 1892). It was only during the last three decades
of the eighteenth century when, as noted already, for the first time in its
known history the infection showed a marked tendency to spread far afield,
that further information on an invasion of contiguous or neighbouring
countries became available.

To judge from the somewhat disjointed and certainly incomplete data
assembled in regard to this period by Macnamara, in 1770 cholera was
endemic in the Arcot region inland from Madras as well as throughout
the Travancore area to the south-west. From 1772 to 1782 the presence of
epidemics was noted on the Coromandel coast. In March 1781 cholera was
prevalent in the Ganjam district in the north-east of the province of Madras,
and attacked within a few days 1143 men out of some 5000 Bengal troops
marching through this area. According to a report on this visitation
dispatched from Calcutta to the Court of Directors of the East India
Company in London, as quoted by Macnamara,

“the disease ... has not been confined to the country of Ganjam; it afterwards found
its way to this place [Calcutta]; and after chiefly affecting the native inhabitants, so as
to occasion a great mortality during the period of a fortnight, it is now generally abated and pursuing its course to the northwards”.

As a consequence, cholera broke out in April 1783 at Hardwar, situated in the Uttar Pradesh (formerly the United Provinces) on the right bank of the Ganges, and apparently killed in less than eight days 20,000 of the pilgrims assembled at that holy place. At the same time the disease raged among the Mahratta armies engaged in war with Tippo Sultan.

That this outbreak of cholera did not hold sway only in India is proved by reports, quoted by Macnamara, which showed that (a) in March 1782 the disease was raging in epidemic form at Trincomalee in Ceylon, severely affecting the British fleet at anchor in this port, which had probably suffered from cholera on a previous occasion already, and that (b) during 1783 cholera existed in Burma.

Statements made to the effect that in 1775 cholera had reached Mauritius or, as claimed by Fabre & Chailan, the nearby island of Réunion (then called Bourbon Island), are open to considerable doubt.

Dealing with further developments, Macnamara summarized that in 1787 and again in 1794 cholera caused terrible ravages in Arcot and Vellore, while in 1790 it was once more prevalent in Ganjam. Information on the years following, up to 1817, is scanty but, to judge from the occurrence of cholera cases among the European troops recorded by the Bengal Medical Board, cholera manifestations continued to occur in various parts of India, including, besides Bengal (where a violent outbreak appears to have taken place in 1814), also Bihar and Orissa, and the Madhya Pradesh (formerly the Central Provinces) as well as the Uttar Pradesh. Supplementing this information, Sticker, besides referring to an outbreak at Travancore in 1792, also noted a further invasion of Ceylon in the year 1804.

Incomplete or even fragmentary though the evidence brought forward above often is, it leaves no room for doubt that cholera, present in India since ancient times, not only continued to exist but was apt to manifest itself periodically in widespread conflagrations. Further, as aptly pointed out by Sticker, even at this early stage one can clearly perceive the ominous role played in the propagation of the disease by military operations and by pilgrimages, when ample fuel became available for the spread of an infection either met with en route to the places of assembly or pre-existent there. For the reasons adduced above it is not surprising, on the other hand, that the known early history of cholera in India furnishes hardly any clue for the cardinal epidemiological importance of Bengal which, according to the present state of our knowledge, has to be considered as the cradle, if not the original home, of the infection. However, as will be discussed now, observations made in that area from 1817 onwards filled this gap in the knowledge of cholera epidemiology in so dramatic a manner that some of the observers were led to believe that the disease had then arisen in Bengal de novo.
First Pandemic (1817)

Untenable though this contention is, it must be admitted that in 1817 a new epoch in the history of cholera began, because this year marks the onset of the first of a series of pandemics during which the infection, after having gained impetus in India through a particularly severe and widely-spread incidence, extended its sway to other parts of the world, paying heed neither to distances and natural obstacles nor to vain attempts at warding off its attacks through cordons and other quarantine measures. One may claim, therefore, that cholera which, as far as is known, had hitherto been of more or less localized importance only, began to become a most serious concern of the world in 1817.1

### TABLE I. CHOLERA PANDEMICS IN THE NINETEENTH CENTURY

<table>
<thead>
<tr>
<th>According to</th>
<th>Haeser (1832)</th>
<th>Hirsch (1833)</th>
<th>Sticker (1912)</th>
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<td>1817-23</td>
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<tr>
<td>4</td>
<td>1863-73</td>
<td>4</td>
<td>1863-75</td>
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* Kolle & Prigge (1928) stated that the 5th cholera pandemic (corresponding to Sticker's 4th) lasted from 1883 to 1896, and the 6th from 1902 to 1923.

It was probably not accidental that the onset of the first cholera pandemic fell within a period during which abnormal meteorological conditions prevailed. In India, in particular, the year 1815 and still more that of 1817 had been marked by extremely heavy rainfalls followed by disastrous floods and harvest failures, while the year 1816 had been extraordinarily hot and dry (Sticker, 1912). Whether *propter hoc* or *post hoc*, it is certain that in 1817 cholera began to show an unusual violence in India. As claimed with much reason by Sticker, this storm probably started in the hinterland of Bengal between the Ganges and Brahmaputra, to reach Calcutta early in August, i.e., before the presence of a “new” disease, called “morbus oryzeus” as it was ascribed to the consumption of spoiled rice.

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1 As will be gathered from Table I above, which illustrates the views held by different writers regarding the dates of onset and duration of successive pandemics, Haeser places the beginning of the first of these in 1816. However, there is no convincing evidence in favour of this view, which is not shared by other authorities.
rice, had been reported on 23 August by Tytler, the civil surgeon of Jessore, a town situated some 50 miles (80 km) north-east of Calcutta on a branch of the Ganges. That this was the real course of events is well shown by the reply to a report from Jessore given by the Calcutta Medical Board which, as quoted by Macnamara, stated in part:

"that the disease is the usual epidemic of this part of the year . . . It is understood that in certain quarters of Calcutta a similar epidemic prevails: and it is probable that there is no considerable town in the low and humid climate of Bengal that is at present entirely free from its operation."

That the outbreak, present at the time in Calcutta and soon officially designated "cholera morbus", nevertheless showed extraordinary features, is proved by a statement made on 17 September 1817 by the Calcutta magistrate, wherein he said that the disease had:

"of late been far more fatal than at any former period within the recollection of the oldest inhabitants, running its course generally in a few hours and sometimes in a few minutes."

The extraordinary virulence of the 1817 outburst is also well demonstrated by the fact stated by Macnamara that:

"within three months from its appearance the disease had been generated throughout the Province of Bengal, including some 195,935 square miles [about 507,500 km²], and within this vast area the inhabitants of hardly a single village or town had escaped its deadly influence."

The Bundelkhand, an area lying between what were later the United and Central Provinces and corresponding to present-day Vindhya Pradesh, was also overrun by the infection. The terrible toll which the disease exacted from the army of the Marquis of Hastings camping in that area is well illustrated by the following entry which, as quoted by Macnamara, the general made in his diary under 17 November:

"The march was terrible for the number of poor creatures falling under the sudden attacks of this dreadful infliction, and from the quantities of bodies of those who died in wagons and were necessarily put out to make room for such as might be saved by the conveyance. It is ascertained that above 500 have died since sunset yesterday..."

In 1818 cholera not only reappeared with undiminished violence in the places where it had raged previously, but rapidly extended in various directions, thus spreading north-eastwards into Nepal, directly or indirectly from the Bundelkhand over Agra and Delhi towards the Punjab, which was eventually reached by the infection in 1820, as well as to Surat and to Bombay and in a southerly direction to Hyderabad, Bangalore, and Seringapatam. Spreading from Ganjam, the infection also reached Madras and Madura.

While the disease continued to be active in 1819 and 1820, it tended to become localized in 1821. In the following year, according to Macnamara,
“the great epidemic which had arisen in 1817, well nigh covering India within the three succeeding years, had now subsided.” In the meantime, however, cholera had become widely spread beyond the confines of the sub-continent.

Bearing in mind that Burma and the island of Ceylon had suffered from cholera even in the past, it is not surprising to find them involved in the widespread outbreaks starting in Bengal in 1817. As claimed by Sticker, Trincomalee was revisited by the disease in December 1818, but, according to Macnamara, the infection did not gain a foothold in Ceylon before 1819, when the ports of Jaffnapatam and Colombo became invaded. From there cholera spread inland, attacking not only the capital of Kandy, but extending “well nigh over the length and breadth of the island”.

To judge from scanty information, Burma and possibly also Siam were invaded by the land route in 1819 (Hirsch, 1883). Bangkok, the capital of the latter country, became infected by the sea route in 1820, the whole country afterwards becoming devastated by the disease. Sea-borne cholera broke out in Malacca in 1820, followed by epidemics in Penang and Singapore.

As was inevitable, the infection also spread to Java, Borneo, and other islands of the Indonesian archipelago, where it became manifest in 1820 or, according to Hirsch (1883), even in 1819. The sufferings of Java were particularly great, 100,000 people succumbing on the island, including 17,000 in Batavia alone. While the Moluccas, said to have been infected through ships from Calcutta, were possibly invaded as late as 1823, cholera had already entered the Philippines in 1820 by way of Manila.

Dealing with the appearance of cholera in China, Wu Lien-teh (1934) maintained that the confines of the country had been reached by the land route as early as 1817. Be this as it may, it is certain that the disease actually invaded China in 1820 via the sea route from Burma and Bangkok. After Canton had become first involved, the infection also became manifest in the same year in the ports of Wenchow and Ningpo and spread into the Yangtze valley. The north of the country became invaded in the following year. Outbreaks in central and northern China, including Peking, recurred during the period 1822-24. It is of interest to add that, according to a statement made by Huc, it is probable that cholera, proceeding from Peking, crossed the Great Wall and followed the caravan route to Kyakhta, thus reaching the Russian border.

The disease made its first appearance in Japan in 1822, having been imported into Nagasaki by a merchant-ship from Java (Takano, Ohitsubo & Inouye, 1926). The infection rapidly extended to Osaka and some other cities, where it exacted a terrible toll in lives.

The cholera invasion of Arabia taking place in the course of the first pandemic stands in causal connexion with the landing of a British expeditionary force sent early in 1821 from India to Oman. The infection, which
first gained a foothold in Muscat, afterwards extended over the greater part
of the territory and subsequently reached Bahrein to the west of the Persian
Gulf as well as Bushire on its eastern shore, thus entering the territory of
present-day Iran. Spreading inland from there, cholera successively invaded
Shiraz and Teheran, finally reaching Resht, situated on the southern shore
of the Caspian Sea.

As was inevitable, cholera also appeared in 1821 at Basra, the principal
port at the head of the Persian Gulf, and killed in less than three weeks
between 15,000 and 18,000 people. The infection was carried up the Tigris
by boat and caravans and, reaching the region of Baghdad, caused ter­
rible havoc in the Persian army which attacked this city at the time. Sub­
siding during the winter, cholera broke out once more in the spring of
1822 along the Euphrates as well as the Tigris. As vividly described by
Macnamara, a Persian army, which had defeated the Turks near Erivan
and had pursued the enemy westwards, fell a prey to cholera. The victors
retreated to Khoi in Iran where they dispersed, disseminating the infec­
tion throughout the country. As a result the disease spread northwards,
reaching Tiflis (now Tbilissi), between the Caspian and Black Seas, and
Astrakhan on the Caspian Sea which, however, had been reached already
by water-borne infection from Resht. Whether these invasions took place
in 1823, as stated by Haeser (1882) and Hirsch (1883), or in 1822 with
recrudescences in 1823, as Macnamara (1876) seems to imply, is difficult
to decide.

That the infection which had thus reached European territory, did not
become entrenched and progress farther was, in the opinion of Sticker, due
to the severe winter of 1823-24 rather than to the feeble control measures
taken by the Russian authorities at Astrakhan. Sticker supported this view
by pointing out that cholera also disappeared from the Tiflis area, where
no preventive work had been done.

Besides spreading in the manner described above, cholera was also
carried by caravans into Syria, reaching Aleppo in November 1822. It
broke out in 1823 at Alexandretta (Iskenderun) and spread along the
Syrian border of the Mediterranean, but entirely disappeared from this area
by the end of the year.

In addition to this more or less continuous spread, cholera made, in the
course of the first pandemic, two long-distance sprints:

(a) The infection appeared at the end of October 1819 in Port Louis,
Mauritius, evidently as the result of an importation by a ship from Trinco­
malce, Ceylon, on which cholera has broken out en route. Three weeks after
the arrival of the vessel, which had landed some of her patients, the disease
became epidemic on shore and claimed over 6,000 victims, mostly Negro
slaves. In spite of the precautions taken, the infection also invaded Bourbon
Island (Réunion) where, however, only 187 casualties resulted.
(b) As recorded by Haeser (1882),

"in the course of its progress to Arabia, the epidemic [cholera] reached during the years 1820-21 also for the first time the near-by coast of Africa, but—to judge from very scanty information—spread only on the narrow coastal zone of Zanzibar (from the 4th degree northern latitude to the 6th degree southern latitude)". [Trans.]

This invasion, which was confirmed by Hirsch (1883) and by Clemow (1903) is not surprising in view of the dense traffic of Arabian dhows between Arabia and the East African coast—a route by which *Xenopsylla astia* was also carried to the latter area (Pollitzer, 1954).

Summing up his description of the first cholera pandemic, Macnamara pointed out that

"the disease absolutely disappeared from Persia, Ceylon, Burmah and China, after existing in these localities for three or four successive seasons—in fact, the epidemic cholera which had extended from India over these countries had again subsided into its endemic area in Lower Bengal—the Home of Cholera, as Dr. Macpherson calls it".

**Second Pandemic (1829)**

Divergent opinions were held in the past regarding the origin of the second cholera pandemic. It was believed in some quarters that it was due to a recrudescence of the infection which had persisted at Astrakhan since the time of the first pandemic. However, it would be impossible to reconcile with this assumption the fact that, before cholera became manifest at Astrakhan in 1830, it had already appeared in 1829 at Orenburg (now Chkalov).

Dealing with the history of cholera in China, Wu Lien-teh (1934) noted that in 1826 the infection was "again borne from India to China; reaching Peking once more and steadily advancing, it crosses the Chinese wall, sweeps through Mongolia and eventually travels to Moscow".

However, while this surmise might explain the appearance of cholera at Orenburg, it could not account for the second inroad of the infection to the west of the Caspian Sea. Little doubt can exist, therefore, that, as advocated by Macnamara, the second as well as the first cholera pandemic can be traced back to Bengal, where the infection had shown signs of increased violence and activity in 1826. This was followed still in the same year by a steady progress of the disease westwards along the Ganges and Jumna rivers and in 1827 by an invasion of the Punjab. While information for 1828 is indefinite, it is known that in 1829 cholera was rampant in Afghanistan, penetrated into Persia, and was also present in the region of Bukhara and Chiva. From there the infection was evidently carried by caravans to Orenburg in the south-east corner of European Russia, where an epidemic broke out at the end of August 1829, and from where cholera soon started to spread north-westwards.

The infection seems to have subsided in Persia during the winter of 1829-30 but became active again in the spring of the latter year. Spreading northwards, it once more reached Resht as well as Baku on the Caspian
Sea, and also reappeared at Tiflis and Astrakhan. As maintained with much reason by Macnamara, it is probable that "the stream of cholera, which entered Russia from the northern provinces of Persia, formed a junction with that which flowed through Orenburg". What is certain is that cholera, which early in 1830 had come to a temporary halt in the Orenburg area, began in the spring an advance on a wide front which ultimately resulted in the invasion, not only of most parts of Europe, but also of large parts of the Americas, as well as of Arabia and East and North Africa. The main features of this truly pandemic spread of the scourge, which alone can receive attention within the scope of the present chapter, will now be described.

Though every possible effort was made by the authorities to stem the tide with the aid of cordons and other rigid quarantine measures, cholera steadily advanced into Russia, reaching Moscow by the autumn of 1830. There was a lull during the winter of 1830-31, but in the spring of the latter year cholera was again in full advance progressing (a) into the Baltic provinces and to St. Petersburg (now Leningrad), to spread from there into the north-western provinces of Russia as far as Archangel on the White Sea, as well as into Finland, and (b) into Poland, where the infection became entrenched among the Russian, and afterwards also among the Polish, troops at war in that country. There can be no doubt that, as emphasized by Haeser (1882) and other authorities, the presence of cholera among these troops has to be considered one of the main causes for the further spread of the infection westwards. In fact, the situation in the Austrian province of Galicia became serious only after it had been entered by Polish and Russian contingents.

From Galicia cholera passed into the interior of Austria, Vienna becoming affected in August 1831. Before that time (in June 1831), Hungary had already been invaded, and here the disease raged with particular violence (Haeser). Outbreaks reappeared in Vienna and some other parts of Austria in 1832.

In spite of the most rigid quarantine measures it proved impossible to prevent the invasion of Prussia, the less so because, inter alia, the infection was carried by a ship from Riga to Danzig. Spreading into the interior of Prussia, the wave of infection reached Berlin in August 1831, while Hamburg became involved in October. In several of the localities then affected in Prussia, including Berlin, and also in Hamburg, cholera became recrudescence in the spring and summer of 1832. A limited outbreak, commencing in August of that year in the Rhine province (Rhineland-Palatinate), was evidently due to an importation of the infection from the Netherlands and not from the east.

The close shipping connexions existing between the Baltic and German ports on the one hand and England on the other made the importation of cholera into the latter country well-nigh unavoidable. In fact the disease appeared in June 1831 on board some warships anchored in a creek of the Medway below London, where vessels coming from Riga were in qua-
rantine. In October of the same year a cholera epidemic became manifest in the port of Sunderland on the east coast of England, but it could not be ascertained how or even when this outbreak had originated. As noted by Macnamara, the disease afterwards appeared at Newcastle, Gateshead, Edinburgh, and, in February 1832, at London, the death toll in England amounting in November 1831 to 97, in December to 282, in January 1832 to 614, in February to 708, in March to 1519 and in April to 1401. Cholera recurred in England during the latter part of 1832 and visited, before the end of August, Hull, York, Leeds, and several other large towns. The total number of cases in 1832 seems to have been 14,796, with 5432 deaths (Haeser; Macnamara).

Cholera appeared in Dublin, Ireland, at the end of March 1832, and spread to many principal towns of that island.

Considering that, until the end of 1831, cholera in Germany had been practically absent from the regions west of the Elbe river and that the outbreaks in England had not assumed large proportions, it is not surprising to find that France up to then remained free from the infection. However, in the middle of March 1832 the disease appeared in Calais and soon afterwards in Paris. Cholera afterwards spread over the greater part of France, only 35 of the 86 departments remaining completely free, mostly those in the southern and eastern mountainous areas.

Cholera appeared in Belgium in the spring of 1832 (first in a village near the French border) but claimed not more than 7984 victims. The disease seems to have caused also comparatively little havoc in the Netherlands, where it first appeared at Scheveningen in June 1832.

In the autumn of 1832 the presence of the infection was also recorded in Norway at Drammen, Moss, and Christiania. Cholera was more widely spread in Norway during the following year, but it was only in 1834 that severe epidemics took place (Hirsch, 1883).

Besides showing a more or less contiguous spread in Europe, cholera also reached, in 1832, the distant shores of America; it was first imported through the agency of ships from Europe which had been quarantined at Grosse Island a few miles below Quebec in Canada. Cases appeared in Quebec early in June and during the following two weeks 1000 cholera deaths occurred in that city. The disease spread with great rapidity along the St. Lawrence River and its tributaries into the interior.

At about the same time the infection was also imported into the United States of America, where it appeared at New York on 23 June and at Philadelphia on 5 July. Continuing to be rampant until 1834, cholera caused great ravages in the country, even spreading, according to Haeser (1882) and Hirsch (1883), across the Rocky Mountains to the Pacific coast. A serious recrudescence of the infection in New York and other centres on the east coast in 1834 seems to have led to the invasion of Halifax in Canada.
In the course of the second pandemic cholera also penetrated into other American countries. As claimed by Haeser, it appeared as early as 1832 in Peru and Chile, but the reliability of this information is denied by Hirsch. Certain it is that in the spring of 1833 the infection became manifest in Mexico, where the high plateau, as well as the coastal, areas became involved. In the same year cholera, apparently imported from Spain, caused serious ravages in the island of Cuba. A recrudescence of the disease there in 1835 led to a further invasion of the USA where, however, besides New Orleans, the portal of entry, only Charleston in South Carolina became affected (Hirsch).

While the appearance of the disease in the coastal areas of Guiana did not lead to serious consequences, a devastating outbreak took place in 1837 in Nicaragua (Haeser). As added by Hirsch, cholera appeared in the same year also in Guatemala.

Though on the whole somewhat relenting in ferocity, cholera continued to reappear in 1833 in some of the formerly affected European countries, e.g., in Hungary, and even to spread to hitherto unaffected areas. Thus the infection was imported early in the year into Portugal through a steamer which, carrying British troops, had left England at the end of December 1832 and had had some cholera deaths en route. Cholera, which broke out at the fort on the mouth of the Douro where the troops had been landed, soon spread, reaching Lisbon early in April 1833.

In spite of quarantine measures enforced with truly Draconic severity in Spain, cholera managed to penetrate into the country in August 1833. Remaining limited during this year, the infection became widely spread in 1834 and even progressed at the end of the year into southern France (Marseilles and other places in Provence). Likewise the disease was carried from Spain to the opposite shore of Africa, particularly to Ceuta.

Another important event of the year 1834 was a serious visitation of Sweden which, as claimed by Haeser and Hirsch, had hitherto remained free from cholera.

When dealing with the cholera manifestations in Europe during the earlier part of the second pandemic, it is not easy to decide how soon the north-eastern part of the Balkan peninsula (i.e. present-day Romania and Bulgaria) had become invaded. According to Macnamara an extension of the infection from southern Russia to these areas occurred as early as 1830, whereas Haeser and Hirsch recorded that they were invaded early in 1831 after the appearance of cholera in the Austrian province of Galicia. Haeser added that at the end of July of that year an epidemic broke out at Constantinople (Istanbul), from where the infection was imported into Smyrna and other places in Asia Minor.

Before dealing with the developments in Europe during the terminal years of the pandemic, attention has to be devoted to an ominous westward spread of the infection from Persia, the invasion of which in 1829 has been
noted above. While Macnamara maintained that even before that time (? 1827) cholera had broken out among the troops of Said-bin Sultan engaged in an attack on Bahrain, according to Haeser it was only in 1830 that the infection progressed from Persia to Mesopotamia and Arabia, where plague was present at the same time. In 1831 cholera, which previously seemed to have been sporadic in Mecca, broke out among the pilgrims assembled at this place, killing nearly one half (? 12,000) of them.

There can be little doubt that those of the pilgrims who were able to return to their homes in Syria, Palestine, and Egypt were responsible for the importation of cholera into these countries. Appearing in Egypt first at Cairo (July 1831), cholera raged with the greatest violence, penetrating up the Nile as far as Thebes as well as invading Alexandria and the whole delta of the Nile. Returning pilgrims were probably also instrumental in carrying the infection to Tunisia, where cholera broke out soon after it had appeared in Egypt.

While cholera seemed to show signs of a decline in Europe during the year 1834, in 1835 it again became rampant in several parts of the continent. As noted already, the infection had been carried at the end of 1834 into Provence. The resulting epidemic in Marseilles on 7 December terminated at the end of March 1835. However, in June a second and far more violent outbreak commenced, at the aeme of which (24-26 July) 1500 persons succumbed. The disease also raged at Toulon and many other places in southern France.

Before dealing with the most serious consequences of this recrudescence of cholera for other parts of Europe, it should be mentioned that at the end of 1834 and much more markedly in 1835 cholera became manifest among French troops sent to Algeria. The civilian population became involved and the infection penetrated deep into the hinterland. According to Hirsch, cholera was again “disastrously prevalent” in Algeria in 1837.

During the period of 1835-37, cholera also displayed great activity in Egypt and appeared in Tripolitania and Tunisia as well as south of Egypt in the Sudan and Abyssinia. The disease also reappeared in 1836-37 on the Somali coast and in Zanzibar.

Considering that (a) cholera raged with great ferocity on the Malabar coast of India in 1833-34 and (b) the disease was present in epidemic form at Mecca during the 1835 pilgrimage, Macnamara postulated with much reason that these cholera manifestations in north-eastern and East Africa were due to a fresh importation of the infection from India. He even claimed that the same held true in regard to the developments in Europe during the period of 1835-37, but one must agree with Haeser that enough remnants of the infection had been left in that continent to account for the recrudescence or spread of cholera.

It should be noted in this connexion that the infection progressed through the Riviera from France into Italy and spread in the latter country
from 1835 to 1837. At the end of this period (1837) the disease appeared also in the Maltese islands. From upper Italy cholera penetrated in 1836 into the Tessin canton of Switzerland and into the Tyrol. A few places in Istria, Croatia, Dalmatia, Carnolia, and Styria also became affected at the same time.

A serious epidemic recurred in Vienna and cholera spread from there into the northern parts of the Austrian Empire and also into Hungary.

From Tyrol the infection penetrated into Bavaria, reaching Munich in October 1836. In the same year there occurred an outbreak at Coventry in England, and cases on a warship anchored near Greenwich.

In the summer of 1837 there were recurrences of cholera in Prussia, Hamburg, and Poland. In the following year no more epidemics developed in Europe, but here and there sporadic cases still occurred.

Information regarding the inroads of cholera into the countries east of India during the second pandemic is scanty. Haeser remarked in this connexion that the infection which had been introduced during the first pandemic into the Dutch East Indies (now Indonesia) and the Philippines, persisted there until 1830, and also claimed that in 1832 cholera reached the Swan River region of Australia, but showed no tendency to spread there. In the opinion of Hirsch, however, “the statement that cholera prevailed on the west coast of Australia (Gaz. méd. de Paris, 1832, p. 499), rests upon hardly reliable newspaper information”.

The Straits Settlements suffered from epidemic cholera in 1826, but then remained free until 1840. As noted before, cholera was reintroduced into China in 1826. In the following year the disease was said to be present in Chinese Tartary, while in 1835 an outbreak (presumably due to a recent introduction from India) was recorded at Canton. According to Hirsch cholera reappeared in Japan in 1831.

While fairly quiescent in India during the years 1835 and 1836, cholera became prevalent in Lower Bengal in 1837 and then spread westwards as far as Afghanistan where an outbreak in Kabul in 1839 was recorded.

Cholera became rampant once more in Lower Bengal early in 1840 at a time when a large number of troops had been assembled in Calcutta and Madras to embark for active service in China. No doubt can exist that the contingents from Calcutta were responsible for importations of the infection first into the Straits Settlements and then into China, where an initial epidemic broke out soon after landings had been effected on the island of Chushan outside Shanghai in July 1840. The infection soon spread to the mainland, where it persisted for this and the following two years, inflicting, as Macnamara put it, “on the unfortunate inhabitants of the Celestial Empire one of the most frightful visitations of disease to which any nation was ever subjected”.

Besides extending eastwards into the Philippines, cholera, spreading westwards from Canton, started on a long journey, in the course of which many countries were to be devastated.
Progressing along the trade route from Canton to Burma, the infection permeated into the northern part of the latter country in 1842 and branched southwards along the Irrawaddy River towards Rangoon. That at the same time cholera inexorably pursued its westwards course is convincingly shown by the statement of an envoy from Sinkiang (Chinese Turkestan) who told Macnamara that in the year 1844 a malady of the nature of cholera "came from the side of Chiria; that during that summer it attacked all the places on or near the main line of traffic from China; that in Kashgar, Yarkund, Kokand and Bokhara, it killed thousands of people; that it lasted for a few weeks in each place and the people died by hundreds every day . . .".

Thus cholera had progressed once more into the area of Bukhara which, as noted before, had been invaded early in the second pandemic. However, while in 1829 the invasion of this area was due to a direct spread of the infection from India, in 1844 cholera, though originally derived from Bengal, had arrived in the Bukhara area by a long indirect route. More curious still, the evidence assembled by Macnamara leaves no room for doubt that, just as it had made earlier in its course a sidetrack into Burma, so cholera, as soon as it met with other paths leading southwards, penetrated into Afghanistan (where it reached Kabul in 1844) and then into the Punjab, from where it extended in 1845 south-westwards to Karachi and south-eastwards to Delhi.

As stated by Macnamara, cholera, continuing at the same time to follow its main course,

"spread as far west as the town of Meshed before the close of the year 1845, and it burst forth there again with renewed violence in the June of the following year, quickly extending to Teheran and Tabreez, and overspreading the province of Ghilan; before the close of the year it reached as far north as the town of Derbent on the Caspian Sea."

The south-eastern corner of Europe had thus been reached by the pandemic wave. The infection does not seem to have progressed beyond Derbent, a Caspian port north of Baku, during the winter 1846-47. Presumably, however, in the latter year new impetus was given to it through the developments described below, which resulted in a second cholera invasion of Persia.

A serious recrudescence of cholera in Lower Bengal in 1845 had led in the course of the same and the following years to an invasion of Madras and Ceylon on the one hand, and of the Bombay area on the other. Progressing westwards from there, "in the month of May 1846 cholera showed itself at Aden, Mocha and Jeddah and invaded almost the whole of the sea-board of the Arabian peninsula; it even penetrated into the interior of Omaun" (Rigler, quoted by Macnamara).

There can be little doubt that this spread of the infection in Arabia led to a cholera invasion of Persia, the less so as it is definitely known
that the disease had gained an entry into Mesopotamia, reaching Baghdad in September 1846 and then spreading northwards up the Euphrates and Tigris.

As noted above, it was probably due to the added effect of this second invasion of Persia that cholera, which had become latent at Derbent during the winter of 1846-47, not only reappeared in this port in April 1847 and spread along the Caspian shore to Astrakhan and then up the Volga, but also broke out in July at Tiflis and progressed from there westwards to the Black Sea coast and north-westwards across the Caucasus mountains into the interior of Russia. Moreover, progressing possibly up the Ural River, the infection reached the Orenburg area and from there spread rapidly into Siberia to reach Tobolsk "previous to July" (Hirsch).

Before dealing with the further advances of cholera in Europe and subsequently also America, attention must be devoted to a second ominous inroad of the infection farther southwards, which culminated in an epidemic killing more than 15,000 people at and near Mecca in November 1846, the disease having been imported probably from the port of Jidda on the Red Sea and not overland from the east.

The progress of cholera resulting from the above-described invasion of Russia was rapid during the summer of 1847, Moscow being reached in September. Soon afterwards, derived probably from the Black Sea ports, the infection became manifest in Constantinople. However, as was usual even during the periods of the most active spread of cholera, there was a lull during the winter of 1847-48 when, according to Macnamara, Olgopol (a place about 30 miles (48 km) east of the Austrian frontier), and the vicinity of Riga had been reached.

Resuming its march early in 1848, cholera progressed not only in Europe, reaching Norway in the north, the Balkan countries in the south, England, Scotland, and Ireland in the north-west, and Spain in the south-west, but was carried on the one hand to Egypt by way of pilgrims returning from Mecca, and on the other to the USA, reaching Staten Island outside New York, and New Orleans, and continuing to spread—still in the same year—from the latter port far up the Mississippi and also to Texas. Thus, as stated by Macnamara, "between May and December 1848, cholera had extended its influence from Moscow (37°E longitude) to the southern part of the United States of America (90°W longitude)". Moreover, a reappearance of cholera at Constantinople led to the invasion of Asia Minor, Syria, Palestine, and possibly even Persia (Haeser).

Following a comparatively quiet spell during the winter, cholera reappeared in the spring of 1849 over the greater part of Europe. The whole of France became involved, the infection spreading from there into Italy as well as to North Africa (Algeria and Tunisia). The ravages of the disease in England were pathetically described by Farr (1852) thus:

"If a foreign army had landed on the coast of England, seized all the seaports, sent detachments over the surrounding districts, ravaged the population through the summer,
after having destroyed more than a thousand lives a day, for several days in succession, and in the year it held possession of the country, slain 53,293 men, women and children, the task of registering the dead would be inexpressibly painful; and the pain is not greatly diminished by the circumstance, that in the calamity to be described, the minister of destruction was a pestilence that spread over the face of the island, and found in so many cities quick poisonous matters ready at hand to destroy the inhabitants.

Justifying the designation of "America's greatest scourge" given to it by Chambers (1938), cholera also caused widespread ravages in 1849 in the USA, where—owing to the appearance of an epidemic in May of that year—New York City had become a most potent centre for the distribution of the infection. Spreading from there, and also continuing its progress from New Orleans, cholera overran practically the whole of the States lying east of the Rocky Mountains and made inroads into Canada which, however, was also invaded by the sea route directly from Europe. Moreover the infection spread by various routes into Mexico, and was also carried at the end of 1849 by ship from New Orleans to the river Chagres in Panama.

During the year 1850 cholera reappeared in a virulent form in Egypt, and spread from there along the whole coastal area of North Africa. In Europe it was reproduced in most areas which had been visited in 1849 and appeared de novo in Denmark and Sweden in the north, and in the Maltese and Ionian islands in the south. The mainland of Greece was spared on this occasion as well as in 1832 and 1837.

Extensions of the infected areas also took place during 1850 in the Americas. California was reached by ship from Panama to San Francisco and by the overland route to Sacramento. In South America cholera penetrated into Colombia as far up as the plateau of Bogotá and—to judge from somewhat unreliable accounts—also into Ecuador, to become prevalent at Quito (Hirsch).

Besides being prevalent on the American continent, cholera raged in 1850 and again in 1851 with rarely paralleled violence in Cuba and in Jamaica, which then seems to have been visited for the first time. From Cuba the infection was carried in May 1851 to Grand Canary Island, where it caused no less than 9000 deaths, most of them within the space of a few days.

In North Africa in 1851 cholera was a serious menace only in Morocco. Outbreaks in Europe during that year were restricted to Poland, Silesia, and Pomerania, while elsewhere the pandemic seemed to have subsided. Noting, however, that in 1852 the disease not only reappeared in Poland, but spread from there into some of the adjacent provinces of Russia as well as into Prussia, some writers such as Tholozan (1868) and Hirsch incriminated a persistence of the infection in Poland as the cause of the new pandemic spread of cholera commencing in 1852. Still, while it would be wrong to disregard the merits of this contention, there can be no doubt
that much impetus was added to this renewed activity of cholera through a fresh wave of infection starting in India in 1849. The result was that, according to Macnamara,

"at the end of 1852, the inhabitants of the northern and western provinces of Russia were under the influence of the cholera of 1848-49, and the inhabitants of her Caucasian provinces were again subjected to a fresh importation of the disease from western India through Persia."

**Third Pandemic (1852)**

There can be no doubt that during its course as well as at its commencement the third cholera pandemic was the combined result of local recrudescences due to a temporary entrenchment of the infection and of repeated importations of the disease so that, as noted by Macnamara, it was no more possible to trace its course step by step as could be done in the previous outbreaks.

The main features of the third cholera pandemic from 1853 onwards may be described as follows.

Besides raging in Persia and Mesopotamia, as a consequence of an 1852 outburst in India, cholera was rampant in 1853 in the northern part of Europe and also reached the USA, Mexico, and the West Indies.

In 1854 the infection continued to exact a serious toll in some countries of northern Europe, for example, England, but was particularly rampant on the continent in the south. The transport of troops from southern France, effected on account of the Crimean War, was no doubt responsible for the appearance of cholera in Greece and Turkey. In the west the disease not only raged in most parts of the USA and Mexico, and in some of the West Indian islands, but also appeared in Canada and in Colombia on the northern shore of South America. The only consoling feature amidst the calamities caused by the infection in 1854, one of the worst cholera years on record, was that observations made in England clearly showed, to those who were not obsessed by fanciful theories, that contaminated water played a major role in the spread of cholera and that consequently a supply of safe drinking-water was of cardinal importance in the prevention of the disease.

Besides reappearing in 1855 in many of the areas affected during the previous year, cholera, which had probably gained impetus through a most serious recrudescence in India, appeared in countries hitherto not, or not seriously, affected during the pandemic. In the Near East the infection spread via Arabia into Syria and Asia Minor. In Africa the disease appeared in Egypt, spread into the Sudan and along the north coast as far as Morocco, and also visited, for the first time, the Cape Verde islands. In Europe the infection penetrated into previously unaffected parts of Italy and adjacent parts of Austria and made an inroad into Switzerland. North America was apparently free, but cholera broke out in Venezuela and Brazil.
Except in Spain and Portugal (including Madeira), cholera did not cause much havoc in Europe during the period 1856-58. However, the disease was rampant during these years in India, where spread of the infection was fomented by the disturbances of the mutiny and the subsequent military operations.

Cholera which, commencing an eastward spread early in the pandemic, had reached Indonesia in 1852 and China and Japan two years later, became most serious in these two empires during the period 1857-59. The Philippines were revisited in 1858, while Korea suffered from the disease in the following year.

Other noteworthy events of the period now under review were (1) four outbreaks of cholera from 1854 to 1862 in Mauritius, and one (1859) in Réunion; and (2) serious inroads of the infection into East Africa where, Zanzibar serving as the main distributing centre, the infection spread along the coast to Mozambique in the south and from there to Madagascar and the Comoro Islands, as well as inland into Uganda. As added by Haeser and Hirsch, cholera, which had already invaded Abyssinia (Ethiopia) in 1853, reappeared there in 1855 and, more markedly, in 1858.

In the Americas cholera manifestations were recorded in 1856 in various parts of Central America, and during that and the following year also in Guiana.

In 1859 cholera showed signs of a much increased activity, ushered in by a serious recrudescence of the infection in Bengal. From India the disease spread, following its old routes, westwards into Persia, Mesopotamia, and Arabia, and in a north-western direction into Russia. It is uncertain, however, to what extent the outbreaks subsequently taking place in that country, as well as in other parts of Europe (Sweden, Denmark, Mecklenburg-Schwerin, western Prussia, the Netherlands, and Spain) were due to this fresh importation or to local reactivation of latent infections. Probably being imported from Spain, the infection appeared in 1859 also in some ports of Morocco and Algeria.

Apart from a serious recrudescence in Spain in 1860, in the course of which Gibraltar became involved, and slight cholera manifestations in St. Petersburg, where the infection seems to have lingered on until 1864, Europe seems to have become free from cholera at the end of 1859.

**Fourth Pandemic (1863)**

The fourth pandemic, beginning in 1863 and lasting, according to Haeser until 1873 or, as maintained perhaps more appropriately by Hirsch and Sticker, until 1875, stood in marked contrast to the previous pandemics because, as summarized by Haeser,

"cholera did not penetrate into the heart of Europe as previously over its ancient paths through Persia, the Caspian sea ports, etc., but by new traffic routes which had been created
in the meanwhile: over Arabia into Egypt, Constantinople, southern France and Italy”.

Opinions as to how and when Mecca was reached by cholera from India during the initial stage of the pandemic were at variance. It was claimed that the disease had been brought to Arabia by pilgrims reaching Jidda by ship from India and even Malacca, but Macnamara, while not denying that this might have been the case, declared that “to attach undue importance to such incidents to the neglect of those broader features presented by the disease in its course from Bengal into Arabia and the Hadjiz, is to complicate the subject, and tends to withdraw our attention from the major to minor details in the history of this remarkable epidemic”.

Whether cholera was already present in Mecca at the time or was imported in 1865 only, it is certain that conditions for a rapid spread of the infection were particularly favourable in that jubilee year, when extraordinarily large numbers of pilgrims were assembled. The outbreak taking place in May 1865 was, therefore, of extreme violence, Macnamara stating that probably, including those who succumbed at Jidda, not less than one third of the 90,000 pilgrims assembled at and near Mecca fell victims to the disease.

The infection was carried from Mecca by returning pilgrims to other parts of Arabia, Mesopotamia, Syria, and Palestine as well as—most fatefully—by the sea route to Suez which was by then connected with Alexandria by a railway. As a consequence cholera broke out in the latter city at the end of May or early in June. Though the epidemic ensuing there was not particularly severe, Alexandria became a distributing centre from where the infection was carried by refugees into other parts of Egypt and by steamer to several Mediterranean ports, among which Istanbul, Smyrna, Ancona, and Marseilles became the most important subsidiary distributing centres.

From Istanbul, which had already been reached in July 1865, the infection spread over Turkey as well as southwards to Asia Minor, Cyprus, Rhodes, and some of the Ionian islands, and north-westwards into Bulgaria, Romania, and apparently also into the (then) Austrian province of Bukovina.

Russia was invaded by different routes from the south but nevertheless suffered little in 1865 and early in 1866, the infection remaining restricted to six governments.

However, having entered through Ancona, cholera became serious in southern Italy, including Sicily. The infection also became fairly widespread in France, where Paris became affected in September 1865, but there were only about 10,000 victims in the whole of the country. Persisting through the winter, cholera reappeared in 1866 in many parts of France. In 1867 only a few of the formerly affected districts suffered to a slight extent.
Spain, infected in July 1865 by a traveller arriving in Valencia from Alexandria via Marseilles, suffered appreciably, but the disease became sporadic in 1866 and then disappeared. An extension of the infection from Spain into Portugal led to outbreaks only in a few places. Cholera also did not assume serious proportions in 1865 in England. An invasion of Luxembourg in the same year was of importance in so far as an exacerbation of the situation there in 1866 led to an appearance of cholera outbreaks in the Rhineland-Palatinate and Westphalia in 1866 and 1867.

Curiously the infection also appeared in the autumn of 1865 in Saxony, having been imported by a woman who arrived in Altenburg with her cholera-affected child from Odessa and soon fell a victim to the disease. 468 cases resulted.

While cholera showed but little activity during the winter of 1865-66, the infection flared up once more in the spring of the latter year, thus ushering in a season which Haeser considered one of the most distressing episodes in the history of epidemics. How far the ravages then caused by cholera in Europe were due to renewed importations of the infection from the east and not to local recrudescences is difficult to decide, the more so as the data supplied regarding this question by Haeser and by Macnamara respectively show a marked discrepancy. No doubt can exist that the war waged by Prussia against Austria and her allies, as well as the hostilities between Austria and Italy, exerted a most unfavourable influence on the cholera situation in central Europe.

In Russia cholera extended its sway from the Caucasus as far as St. Petersburg and from Orenburg to the western border of Poland, claiming in 1866 a toll of more than 90,000 lives. The disease reappeared in the spring of 1867, but caused much less havoc.

With the exception of Sweden, which recorded 4503 cholera deaths, the Scandinavian countries suffered little in 1866. In Germany, on the contrary, epidemics breaking out in several regions caused a great loss in lives, the cholera deaths in Prussia alone amounting to almost 115,000. The situation was also most serious in war-torn Austria-Hungary, resulting in a cholera mortality of about 80,000 in Bohemia and Moravia, while other parts of Austria also suffered, and 30,000 succumbed to the disease in Hungary. In Italy there was a serious cholera recrudescence in 1866, for which the military operations were largely responsible. During that year cholera also led to almost 20,000 deaths in the Netherlands and over 30,000 in Belgium. In Great Britain cholera became manifest in many places but usually did not spread, so that the death toll from the disease totalled not more than 14,378, 5,596 succumbing in London, 2,501 in Ireland, and 1,170 in Scotland.

Generally speaking, cholera was far less severe in Europe in 1867 than during the previous year. An exception was formed in Italy, where widespread epidemics, involving even Sardinia, led to 130,000 deaths. Importa-
tions of the infection from Italy led to sporadic attacks or limited outbreaks in Switzerland. In 1868 cholera reappeared in only a few European localities, particularly in Essen, North-Rhine, Germany, and in Reggio di Calabria and Messina, Italy.

Besides raging in Europe and, as will be discussed below, in the Americas, during the period now under review cholera showed an amazingly extensive spread in Africa.

An importation of the infection, apparently from Bombay via Aden, taking place in 1864, led to an invasion of Somaliland, where cholera caused great ravages in 1865.

In February 1865 the infection was carried across the Red Sea from Jidda to Suakin and Massawa and penetrated from there into Abyssinia (Ethiopia). Continuing a southward course, cholera eventually (1869) reached the region of the Kilimanjaro and spread from there in various directions, particularly (a) south-westwards to and across Lake Tanganyika to invade finally, in 1870, the upper reaches of the Congo River, and (b) south-eastwards to Zanzibar island where, in 1869, 70,000 persons succumbed to the disease.

Progressing also from the south end of Lake Tanganyika along trade routes on the western shore of Lake Nyasa, cholera reached, in May 1870, the city of Mozambique. This port, like Zanzibar, became a distributing centre of the infection, which was thus carried to the Comoro Islands, Madagascar, and the Seychelles.

The countries on the Mediterranean shore of Africa, which were also ravaged by cholera during the period under review, seem to have been invaded by various routes. Thus it was claimed that in 1867 cholera was imported into Tunisia by smugglers from Sicily, while the infection of Algeria in 1865 was probably derived from France. The Algerian invasion culminated in an outbreak taking place in 1867 and was alleged to have caused 80,000 deaths.

Similarly Morocco, though already infected through pilgrims returning from Mecca in 1865, had its most violent outbreak in 1868, when the disease, imported from Algeria, seems to have progressed from the hinterland towards the coast.

In 1868 cholera, carried probably by caravans from Morocco, appeared at Podor on the Senegal River in French West Africa and then progressed to St. Louis. From there the infection spread, via MacCarthy Island, to Bathurst in Gambia and Bissau in Portuguese Guinea (1869). According to Macnamara, at Bathurst cholera carried off 1700 victims out of a population of about 5000.

During the period 1865-70 cholera became epidemic in several West Indian islands—first, imported from Marseilles, in Guadeloupe, where it claimed in 1865-66 almost 12,000 victims among a population of about 150,000, then in Santo Domingo (1866), St. Thomas (1868), and Cuba (1867-70).
Whether cholera reached the USA in 1865 or in 1866 is uncertain. Chambers considered it possible that the infection, imported by several ships from Le Havre, appeared at New York in the autumn of the former year, but was soon suppressed by the cold weather. The onset of a serious outbreak in May 1866 might, therefore, have been the result of a recrudescence of the infection and not of its recent importation by cholera-affected ships, particularly the German steamer England, as assumed by Haeser. It is certain that cholera was rampant in New York during the summer and autumn of 1866, the official figure of about 2000 deaths being probably far below the mark.

The further spread of the infection in the USA was facilitated by a considerable extension of the railways into the interior of the country, which had taken place since 1849. An even more ominous role in the spread of cholera there during 1866 was played by troop movements due to the reorganization of the army after the war between the States. Military encampments like that at Newport, Kentucky, thus became subsidiary distributing centres of the infection, in addition to several of the major cities such as New Orleans, where the disease, probably imported by troopships from New York, appeared in July and, lasting until October, claimed a toll of about 1200 lives.

In contrast to previous outbreaks, the role of New Orleans as a distributing centre was limited because, as aptly stated by Chambers, "trains from the Eastern ports outstripped the steamboats to Cincinnati, Louisville, Chicago and St. Louis in carrying the seeds of the scourge, just as they were winning the race for the commerce, travel and romance of the upper interior valley".

However, transport of the infection by ships, particularly by vessels carrying troops, was responsible for the appearance of cholera in several localities of Louisiana as well as of other southern States, including Texas.

The spread of the disease by the railway traffic was responsible for the appearance of cholera in the Middle West as far as Kansas. A solitary infection observed at Albuquerque, New Mexico, indicated, according to Chambers, the western limit of the 1866 invasion.

Though, as estimated by this writer, the number of cholera deaths occurring in the USA during 1866 possibly amounted to 50,000, it deserves attention that, according to him, "even so the mortality in '66 did not compare to that of previous epidemics. While estimates for the whole country were not even attempted for either of the previous epidemics, in '33 a mortality of 5 percent, 10 percent or even 15 percent of the population of a locality was not unusual; the mortality in '49 seldom reached 10 percent; while in '66 we know of no considerable community where the mortality reached 5 percent".

As was to be expected, in 1867 a recrudescence of the infection was observed in many of the principal cities which had suffered from cholera during the previous year. With few exceptions, however, these manifestations were restricted to a few or a limited number of cases. A major outbreak took place at New Orleans, which suffered at the same time from
yellow fever. While the latter disease claimed over 3000 lives, the number of cholera cases was restricted to 575. Some spread of cholera from New Orleans to adjacent territories took place, apparently brought about mainly by troop movements.

While Canada remained almost free from the infection during the period under review, an importation of the disease from New Orleans led to cholera manifestations in Central America (Nicaragua and British Honduras) from 1866 to 1868. At the same time the disease, first becoming entrenched among Paraguayan troops engaged in war against combined forces of Argentina and Brazil in April 1866, reached, in the autumn of that year, the Argentinian city of Corrientes. A rerudescence of the infection there early in 1867 led to a spread of cholera down the Paraná River, in the course of which Buenos Aires was reached in December. In 1868 Uruguay also became affected. Involvement of the interior provinces of Argentina in 1869 led to an overland invasion of Bolivia and Peru, where the disease spread from the hinterland to the coast. As maintained by Hirsch, in contrast to Haeser, this was the first appearance of cholera on the west coast of South America.

In addition to the above-mentioned countries, Brazil became invaded by cholera in April 1867. Entering from Paraguay, the infection spread in the States of Rio de Janeiro and Rio Grande do Sul and again became prevalent in 1868.

While, as noted above, in 1868 cholera became manifest in only a few places in central Europe and the west of the continent remained free, the infection continued to persist during that and the following year in Russia, but did not, as a rule, cause much havoc. A moderately severe epidemic taking place at Kiev in August 1869 was, in the opinion of Macnamara, possibly the result of a reimportation of the infection from Persia, where cholera raged perennially from 1865 to 1871. It is noteworthy, however, that a minor outbreak had already taken place in Kiev in 1868.

Cholera was more active in Russia during 1870, when 37 governments suffered. In the following year the disease raged in practically all parts of European Russia as well as in the Tobolsk and Tomsk governments of Siberia, claiming a total death toll of 130,000. Almost the same mortality was recorded in 1872, when the southern and western governments in particular were involved. In 1873 there were but few outbreaks in Russia proper, but cholera remained active in Poland during that and the following year.

During 1871 cholera spread from Russia in various directions. Southwards the infection was carried to Black Sea ports in Romania and Bulgaria and also to Istanbul and Trabzon in Asia Minor. Manifestations of the disease in other localities of Asia Minor in 1871 and 1872 probably stood in causal connexion with these invasions. Cholera also became prevalent in Romania in 1872 and, more markedly, in 1873, when the infection spread into Bulgaria and from there to a slight extent also to Salonica.
Westwards, cholera spread in 1871 from Russia to (a) Finland and Sweden, where no major epidemics took place; (b) Prussia; and (c) the Austrian province of Galicia.

The infection spread in Prussia during the summer of 1871 as far as Berlin and also reached Hamburg, but except in East Prussia no major outbreaks resulted. While during the year 1872 cholera remained sporadic in the easternmost part of Prussia, major outbreaks, causing a total death toll of 33,156, took place in 1873 in many parts of Germany, including, besides Prussia and Hamburg, Bavaria, Württemberg-Baden, and Hesse. During the winter of 1873-74 cholera remained manifest in Bavaria (particularly in Munich) and in a district of Prussian Silesia, where a major outbreak occurred in the spring of 1874.

Austria had serious outbreaks in 1872 and, to a much lesser extent, in 1873. Hungary suffered severely during these two years, when cholera claimed a total of 190,000 victims.

Though repeated importations of the infection into Great Britain took place during the period under review, it was invariably possible to prevent a spread of the infection. Similarly, the appearance of sporadic cases in the Netherlands and Belgium did not lead to serious consequences. Slight outbreaks were noted in 1873 in Sweden and at Bergen in Norway. In France cholera appeared at Paris as well as in several other districts, a major epidemic developing at Caen.

In the USA, New Orleans and the Mississippi basin once more became seriously involved during the year 1873.

Besides India, where cholera raged with particular violence in 1875 (364,755 deaths), other Eastern territories suffered severely during the concluding years of the pandemic.

An exacerbation of the cholera situation in Persia where, as noted above, the infection had become entrenched since 1865, led to most violent outbreaks in 1870 and to a spread of the infection into Turkish Kurdistan, Mesopotamia, and Arabia.

During 1871-72 the infection, derived possibly from Persia, besides progressing westwards to Egypt, spread in an eastern direction into Bukhara and Russian Turkestan.

A reappearance of cholera at Mecca in 1872 resulted in an invasion of cholera via Suakin into the Sudan.

It also deserves mention that in 1875 Syria was devastated by a cholera outbreak of unknown origin.

To judge from scanty information, the regions in Asia to the south-east and east of India repeatedly suffered from cholera throughout the pandemic now under review. As stated by Wu Lien-teh, in 1862 the disease was widespread in China, reaching Peking and Manchuria. Thousands of people were stated to have fallen victims to the infection in Shanghai.
According to Hirsch, disastrous epidemics, connected probably with the serious exacerbation of the cholera situation in India in 1863, occurred in the “East Indies” (Indonesian archipelago) in 1863 and 1864, and in China and Japan in 1864-65.

Prevalence of the infection in Thailand and Malaya led in 1873 to most serious inroads of cholera into Sumatra, Java, and Madura. From Singapore, which seems to have acted as the main distributing centre, the infection was also carried to Borneo and—directly or indirectly—to Manado on Celebes.

As far as the records collected by Wu Lien-teh go, the incidence of cholera in China was not particularly heavy during the last years of the pandemic. Whether the disease was present at that time in Japan could not be established.

However, most serious outbreaks took place there in 1877-79, in which latter year 158 204 cases with 89 207 deaths were recorded.

Fifth Pandemic (1881)

Although, notwithstanding the wide areas over which it held sway, the fifth cholera pandemic, customarily stated to have lasted from 1881 to 1896, caused considerably less havoc than its predecessors, it marks a most important epoch in the history of this disease. For in 1883-84 Koch, studying the outbreaks then rampant in Egypt and Calcutta, was able to prove that, as had been suspected before by some advanced thinkers, cholera was the result of a specific gastro-intestinal infection.

The main features of the pandemic may thus be outlined.

As the result of a serious exacerbation of the cholera situation in India, which led in 1881 to violent outbreaks in the Punjab, especially in Lahore, the infection was carried to Mecca, where epidemics occurred in that as well as in the following year. In 1883 cholera, possibly already imported during the previous year by pilgrims returning from Mecca (Hussein), became epidemic in Egypt, first at Damietta, situated at one of the mouths of the Nile not far from Port Said, where a fair was in progress at the time. Spread initially by infected persons fleeing from Damietta, the disease broke out in Cairo, Alexandria, and other places, claiming—according to Hussein—58 511 victims in the country.

In Europe cholera remained during the early years of the pandemic practically confined to France, Italy, and Spain. In the first-mentioned country it assumed epidemic proportions in April 1884 at Toulon, and this outbreak was soon followed by small epidemics in other places, including Marseilles and Paris, the total number of cases recorded during the year in France amounting to about 10,000 with a mortality of 50% (Sticker). Cholera reappeared in France in 1885, mainly in localities afflicted during the previous year. In 1887 a small outbreak (7 cases with 4 deaths), due to
the arrival of an infected sailing vessel, took place on the island of Yeu in the Bay of Biscay (in der Beck, 1948).

Though an attempt was made to protect Italy through quarantine measures, cholera became widely spread there in 1884, but caused great havoc only at Naples where, in August and September, over 10,000 cases and more than 5,000 deaths were recorded. The infection persisted in Italy and again became widespread in 1886 and 1887, but no further major epidemics developed.

Spain did not suffer severely from cholera in 1884 (592 deaths), but in the summer of 1885, when the provinces of Valencia and Murcia in particular became afflicted, the case incidence rose to 160,000 with almost 60,000 deaths. The country was once more visited by cholera in 1890.

Though cases were repeatedly imported into Great Britain, the infection invariably failed to entrench itself, both because adequate measures were taken and because wholesome water supplies (eine für alle Zwecke der Reinlichkeit genügende Wasserversorgung (M. Pettenkofer, quoted by Pertl, 1940)) were available.

An importation of cholera into New York by way of an infected steamer arriving in October 1887 from Marseilles and Naples was averted by the rapid establishment of a correct diagnosis through laboratory methods. As maintained by Chambers, this had been the first occasion "to put bacteriology to practical use in combating an invasion by the scourge".

However, although the disease failed to gain entry into North America, serious outbreaks during the period under review took place in South America (Argentina, 1886 and 1888; Chile, 1887 and 1888).

Violent cholera outbreaks in 1892 in Afghanistan and Persia, where the infection had found a temporary home, led to an invasion of Russia via Baku. The infection once more reached Moscow and St. Petersburg and extended to the western confines of the country. Continuing to exist in 1893 and 1894 (when serious outbreaks took place in the Volyniya-Podolsk area), cholera is estimated to have claimed 800,000 victims in Russia during this period.

In 1892 cholera became widespread not only in Russia but also in Germany and France; it assumed serious proportions only at Hamburg, however, where an explosive outbreak, due no doubt to the distribution of unfiltered Elbe water by the waterworks, took place. The incidence of the disease in Hamburg and its suburbs, where this water was utilized, was therefore incomparably higher than that in two adjacent communities obtaining their water supplies from other sources, as is shown by the following data, quoted by Sticker:

<table>
<thead>
<tr>
<th>Locality</th>
<th>Number of inhabitants</th>
<th>Number of cases</th>
<th>Cases per mille</th>
<th>Number of deaths</th>
<th>Deaths per mille</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamburg and suburbs</td>
<td>579,904</td>
<td>19,891</td>
<td>34.3</td>
<td>7,582</td>
<td>13.0</td>
</tr>
<tr>
<td>Altona</td>
<td>143,249</td>
<td>572</td>
<td>3.9</td>
<td>328</td>
<td>2.3</td>
</tr>
<tr>
<td>Wandsbeck</td>
<td>20,571</td>
<td>64</td>
<td>3.1</td>
<td>43</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Cholera appeared in more than 250 other German communities besides Hamburg, but since the cases remained mostly sporadic, the total number in these places was restricted to 1048, with 607 deaths (Sticker). The reappearance of the disease in Germany during the following years also caused little havoc, the case incidence in 1893 being 915 (with 396 deaths) and that in 1894, when the eastern parts of the empire alone were involved, amounting to 1004 (with 490 deaths).

As stated by in der Beeck, cholera appeared in 1892 in the northern departments of France (including Paris and its vicinity) but did not assume epidemic character. In the following year it was mainly the southern parts of the country that were affected, but in most of the 33 departments involved there were only sporadic cases or at most small outbreaks. In 1894 sporadic attacks alone were noted in Toulon, Marseilles, and Paris.

Though, as described by Chambers, eight badly infected ships arrived in New York harbour during 1892, adequate measures, facilitated by the opening of a city health laboratory, rendered it possible to keep the infection at bay, with the result that none of the 10 cases occurring in the city led to the establishment of a focus.

However, as earlier in the pandemic, cholera appeared in South America, involving Brazil in 1893-95, Argentina in 1894 and 1895, and Uruguay in 1895. Still, as stated by Sticker, the infection invariably failed to entrench itself in these countries (es blieb bei kraftlosen Anfängen, die rasch von selber erloschen).

In Africa, according to a table furnished by Kolle & Schürmann (1912), the following countries recorded cholera manifestations during the period under review:

<table>
<thead>
<tr>
<th>Year</th>
<th>Countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>1893</td>
<td>Tripolitania, Tunisia, Algeria, Morocco, French West Africa</td>
</tr>
<tr>
<td>1894</td>
<td>Sudan, Tripolitania, French West Africa</td>
</tr>
<tr>
<td>1895</td>
<td>Egypt, Morocco</td>
</tr>
<tr>
<td>1896</td>
<td>Egypt</td>
</tr>
</tbody>
</table>

However, with the exception of the 1896 outbreak in Egypt, which caused over 16,000 deaths (Hussein, 1949), no considerable epidemics resulted.

Throughout the pandemic, cholera not only continued to be prevalent in India, but appeared frequently or even perennially in the countries to the south-east or east of India. Besides outbreaks in Annam taking place, according to Wu Lien-teh, in 1882, cholera manifestations in South-East Asia were recorded by Kolle & Schürmann thus:

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1 As stated by Simmons et al. (1944), in 1891 cholera occurred in the Setit River region of Eritrea. According to the same authors the last cholera outbreak in Ethiopia occurred in 1892-93.
<table>
<thead>
<tr>
<th>Year</th>
<th>Countries affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1888, 1889</td>
<td>Indonesia (&quot;Sunda Islands&quot;)</td>
</tr>
<tr>
<td>1890</td>
<td>Ceylon, Thailand, Straits Settlements, &quot;Sunda Islands&quot;</td>
</tr>
<tr>
<td>1891</td>
<td>Java</td>
</tr>
<tr>
<td>1896</td>
<td></td>
</tr>
</tbody>
</table>

Cholera was also reported to be present in Thailand and Indonesia during 1897.

In China the infection appears to have been particularly widespread from 1881 to 1883 as well as in 1888 and—to a lesser degree—in 1890 and 1895, while the presence of the disease in Korea in 1881, 1888, 1890, 1891, and 1895 was noted by Wu Lien-teh.

Cholera epidemics in Japan during the period under review took place, according to Takano and co-authors, in 1881 (9000 cases), 1882 (more than 50,000 cases), 1885 (13,772 cases), 1886 (155,000 cases), 1890 (46,000 cases), 1891 (11,000 cases), and 1895 (over 55,000 cases).

An outbreak at Manila in 1882 was mentioned by Hirsch. The presence of the disease in the Philippines was also recorded in 1888 and 1889 (Kolle & Schürmann).

Sixth Pandemic (1899)

The appearance of the sixth cholera pandemic, which may be said to have lasted until 1923, stood, no doubt, in causal connexion with a most marked exacerbation of the cholera situation in India. It is true that, as pointed out by Sticker, after the fifth pandemic the disease had not totally disappeared from western Asia and even Egypt, but a local recrudescence from foci of the infection which possibly continued to persist in western Asia could, at most, have been of auxiliary importance.

This exacerbation of the cholera situation in India, commencing in 1899, led in 1900 to violent outbreaks in Calcutta and Bombay, followed, until 1904, by a prevalence of the disease in the south of the sub-continent, particularly in the Presidency (now State) of Madras, as well as in the north. That the infection possessed from the first a great tendency to spread beyond the confines of India is shown by a westward extension of cholera into Afghanistan and the Persian Gulf areas, taking place in 1900, and by the invasion of Burma and Singapore in 1901 which, as is described on page 45, led to a further spectacular progress of the disease eastwards in the following year.

Simultaneously with this spread to the east, cholera was carried in 1902 by the maritime route, presumably by pilgrims who left Madras, to the port of Jidda and from there to Mecca, where an outbreak beginning in the last week of February killed 4000 of the assembled multitude. Though every possible precaution was taken, it proved impossible to prevent
an invasion of Egypt, where the disease, imported in some manner never elucidated, first became manifest in Asyut and then spread, claiming within three months almost 34,000 victims (Hussein).

In what way the infection penetrated early in this pandemic into Russia is difficult to decide. In the opinion of Sticker, an invasion of Syria, taking place via the Sinai Peninsula in 1903, was responsible for the appearance of cholera in the same year not only in Palestine, Asia Minor, and on the Black Sea coast, but also in Mesopotamia and Persia, from which latter country the disease was imported in the spring of 1904 by caravans via Samarkand into Baku on the Caspian Sea. It is certain that cholera, becoming epidemic in this port in September 1904, spread in the same year still westwards into Transcaucasia, northwards via Astrakhan up the Volga as far as Samara (now Kuibishev), and, according to Sticker, also into western Siberia. In 1905 cholera remained restricted to the valleys of the Ural, Volga, and Don rivers, while the infection seems to have become quiescent in 1906. In the following year, however, the disease once more became epidemic in the Volga basin and spread in 1908 (a) as far as St. Petersburg and some of the Baltic ports; (b) to several Black Sea ports; and (c) eastwards into Transcaisia, Turkestan, and Siberia. The cholera incidence slightly abated in 1909 but, as shown by Table II, rose in 1910 to over 230,000 cases with almost 110,000 deaths, particularly severe epidemics being noted in Jekaterinoslav (18,894 cases), St. Petersburg (45,911 cases), Kiev (40,777 cases), and Orenburg (33,555 cases). Cholera

### TABLE II. CHOLERA INCIDENCE IN EUROPEAN RUSSIA FROM 1902 TO 1925

<table>
<thead>
<tr>
<th>Year</th>
<th>Cases</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>1902</td>
<td>2,167</td>
<td>323</td>
</tr>
<tr>
<td>1904</td>
<td>4,638</td>
<td>846</td>
</tr>
<tr>
<td>1905</td>
<td>498</td>
<td>198</td>
</tr>
<tr>
<td>1906</td>
<td>22</td>
<td>1918</td>
</tr>
<tr>
<td>1907</td>
<td>1,205</td>
<td>624</td>
</tr>
<tr>
<td>1908</td>
<td>58,755</td>
<td>15,642</td>
</tr>
<tr>
<td>1909</td>
<td>231,584</td>
<td>15,677</td>
</tr>
<tr>
<td>1910</td>
<td>352,232</td>
<td>16,635</td>
</tr>
<tr>
<td>1911</td>
<td>3,476</td>
<td>1,548</td>
</tr>
<tr>
<td>1912</td>
<td>1,284</td>
<td>1324</td>
</tr>
<tr>
<td>1913</td>
<td>524</td>
<td>164</td>
</tr>
<tr>
<td>1914</td>
<td>9,715</td>
<td></td>
</tr>
<tr>
<td>1915</td>
<td>36,455</td>
<td></td>
</tr>
<tr>
<td>1916</td>
<td>1,500</td>
<td></td>
</tr>
<tr>
<td>1917</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>1918</td>
<td>41,586</td>
<td></td>
</tr>
<tr>
<td>1919</td>
<td>5,119</td>
<td></td>
</tr>
<tr>
<td>1920</td>
<td>29,015</td>
<td></td>
</tr>
<tr>
<td>1921</td>
<td>201,388</td>
<td></td>
</tr>
<tr>
<td>1922</td>
<td>86,578</td>
<td></td>
</tr>
<tr>
<td>1923</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>1924</td>
<td>1324</td>
<td></td>
</tr>
<tr>
<td>1925</td>
<td>Sporadic cases only</td>
<td></td>
</tr>
</tbody>
</table>

* After Oltzsch (1939)
† No records available
caused no great havoc in Russia in 1911 and appears to have become sporadic during the following two years. However, as shown by the adjoined table, the disease again became widespread during the First World War, particularly in 1915, and, having also been frequent in 1918 and 1920, showed a terrifyingly high incidence in 1921. 1922 was still a bad cholera year, but there was a marked decline in 1923, while only sporadic cases were noted in 1924 and 1925. Since then Europe has remained free from cholera.

The orbit within which the prevalence of cholera during the period under review led directly or indirectly to the invasion of Western countries was far more limited than had been the case in previous pandemics. The infection failing to penetrate into the Americas, the westernmost point reached by the disease was Madeira, which was affected in October 1910 through the arrival of a steamer with unreported cases among immigrants en route from Russia to South America. Lasting until February, this epidemic claimed—according to the official records—600 victims among 1769 patients (Goldschmidt, 1910).

The visitations of western Europe by cholera during the sixth pandemic were restricted to the appearance of sporadic cases or, in the rare instances where a spread of the infection did take place, to abortive outbreaks. Thus, importation of the disease into Rotterdam in 1909 led to only 26 cases with 6 deaths among the population of the port, and to isolated occurrences in 18 other communities of the Netherlands (Sticker).

Though also causing considerably less havoc than on previous occasions, cholera at times during the sixth pandemic assumed quite serious proportions in central and south-eastern Europe. In Italy, where insignificant manifestations had been observed in 1909 in Apulia and at Naples, there were considerable outbreaks during the two years following. In the summer of 1910 the infection, stated to have been recently imported via Brindisi through gipsies coming from Russia, claimed within a few weeks 1400 victims, but, as in 1909, remained restricted to the south of the country. In the summer of 1911 cholera became manifest in all parts of Italy, including Sicily, but assumed serious proportions in only a few of the numerous affected localities.¹

In Hungary, where, as in several other European countries, cholera had been sporadic in 1909, a few epidemics took place in the following year and again in 1913. There, as in Austria, importations of the infection through Russian (and later also through Serbian) war prisoners led to a quite serious cholera situation during the First World War (1914-16). In November 1914 Austrian troops, who had come from the Volyniya-Podolsk area, were instrumental in bringing the infection into Prussian Silesia, but no serious outbreak resulted. However, as in the case of Austria-Hungary,

¹ The prevalence of cholera in Italy was presumably responsible for the appearance of an epidemic in Tunisia in 1911, in the course of which 733 cases occurred.
transports carrying Russian prisoners of war were responsible for the importation of cholera into the interior of Germany, where the disease became manifest in and near prison camps situated in various parts of the country. Still, as stated by Krehnke, the number of cholera victims among the civilian population of Prussia from 1914 to 1918 totalled less than 60. Except among troops stationed in Turkey, the incidence of the disease in the German army, which had been systematically vaccinated against cholera, remained low.

As shown by Table III, cholera outbreaks during the period under review were quite frequent and often serious in the Balkan peninsula, where the spread of the infection was facilitated by the local wars taking place in 1912 and 1913 and also to some extent by the First World War.

<table>
<thead>
<tr>
<th>Year</th>
<th>Countries affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1910</td>
<td>Greece, Turkey</td>
</tr>
<tr>
<td>1911</td>
<td>Bulgaria, Greece, Montenegro, Romania, Serbia, Turkey †</td>
</tr>
<tr>
<td>1912</td>
<td>Bulgaria, Turkey †</td>
</tr>
<tr>
<td>1913</td>
<td>Bulgaria, Greece, Romania, † Serbia, † Turkey</td>
</tr>
<tr>
<td>1914</td>
<td>Bulgaria, Serbia †</td>
</tr>
<tr>
<td>1915</td>
<td>Serbia</td>
</tr>
<tr>
<td>1916</td>
<td>Albania, Bosnia and Herzegovina, † Corfu, Turkey</td>
</tr>
<tr>
<td>1917</td>
<td>Turkey (Istanbul)</td>
</tr>
<tr>
<td>1918</td>
<td>Macedonia</td>
</tr>
<tr>
<td>1919-20</td>
<td>Turkey (Istanbul)</td>
</tr>
<tr>
<td>1922</td>
<td>Greece (Athens), Romania</td>
</tr>
</tbody>
</table>

* Largely based on data from Kolle & Schürmann (1912) and Kolle & Frigge (1928); Greece is included for 1913 on the authority of Savas (1914).
† Major outbreak

In south-west Asia during the period under review cholera manifestations continued to be frequent in Arabia and Persia. A particularly violent outbreak, due, apparently, to the arrival of pilgrims by ship via Odessa, arose in Mecca at the end of 1907 and claimed in 1908 more than 25,000 victims in the Hejaz (Sticker). Further appearances of the disease in Arabia were recorded in 1909 (Hejaz), 1910 (Mecca), 1911 (major outbreak involving Mecca), and 1912. According to Duguet, Mecca and the Hejaz as a whole have remained free from epidemic cholera since then.
In Persia cholera appears to have been rampant in 1906 but seems to have caused no great havoc when reimported from the north in 1908 (Sticker). Further manifestations of the disease in Persia were recorded in 1911, 1912, perennially from 1914 to 1919, and also in 1922-23.

No doubt fomented by the First World War, cholera was rampant in Turkey-in-Asia in 1916. After the war outbreaks were recorded in Mesopotamia in 1918 and 1919 as well as in 1923 (Heggs, 1938), and in Palestine in 1918.

As noted already, the great activity displayed by the infection before the beginning of the sixth pandemic in India led to a rapid spread of the infection south-eastwards and eastwards. The invasion of Burma and Malaya in 1901 was thus followed in 1902 by a spread of cholera over most parts of the Far East as far as China and Manchuria, Korea, Japan, and the Philippines. It is possible, however, that in some of the countries then invaded the new wave of infection merely reactivated already existing cholera foci. Be this as it may, it is certain that in most of the countries involved outbreaks continued to be frequent or even perennial, though varying in extent and severity. As far as can be gathered from the compilations of Kölle & Prigge (1928), Swaroop & Pollitzer (1952), and Wu Lien-teh (1934), particularly serious outbreaks took place as shown in Table IV.

As will be noted, in some of the countries concerned the cholera situation was particularly serious in 1908, 1909 or in both years. It is interesting to note that these bad cholera years were preceded by a period lasting from 1905 to 1908, during which cholera was particularly rampant in India, as shown by the following figures.

<table>
<thead>
<tr>
<th>Year</th>
<th>Cholera deaths in India</th>
<th>Year</th>
<th>Cholera deaths in India</th>
</tr>
</thead>
<tbody>
<tr>
<td>1904</td>
<td>189,855</td>
<td>1907</td>
<td>400,024</td>
</tr>
<tr>
<td>1905</td>
<td>439,439</td>
<td>1908</td>
<td>579,814</td>
</tr>
<tr>
<td>1906</td>
<td>682,649</td>
<td>1909</td>
<td>227,842</td>
</tr>
</tbody>
</table>

The cholera mortality in India once more exceeded half a million annually in 1918 (556,533 deaths) and in 1919 (565,166 deaths). As shown by Table IV, the cholera mortality in Java became quite unusually high during these two years, while 1919 was a bad cholera year for Thailand and China. It is, however, difficult to decide whether these parallel developments indicate more than coincidences.

Conclusion

When trying to deal in a summary manner with the geographical distribution of cholera throughout the world, it is far easier to refer to the few areas unaffected by this scourge than to enumerate the many countries where the presence of the disease has been recorded. Generally speaking, it may be maintained that the infection has not penetrated into the
### TABLE IV. YEARS OF HIGH CHOLERA INCIDENCE IN SOUTH-EAST ASIA

<table>
<thead>
<tr>
<th>Year</th>
<th>Burma</th>
<th>Indo-China</th>
<th>Thailand</th>
<th>Federation of Malaya</th>
<th>China-Korea</th>
<th>Japan</th>
<th>Philippines</th>
</tr>
</thead>
<tbody>
<tr>
<td>1902</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1903</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>6,067</td>
<td></td>
</tr>
<tr>
<td>1904</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1905</td>
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<td></td>
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<tr>
<td>1906</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1907</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1908</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1909</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1910</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1911</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1912</td>
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</tr>
<tr>
<td>1913</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1914</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1915</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1916</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1917</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1918</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1919</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1920</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1921</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1922</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Where mortality records are available, figures are given.
northernmost and southernmost parts of the globe. Accordingly, it may be noted that in Asia, northern Siberia and Kamchatka have been spared and the same holds true of the most northern parts of western Europe (Iceland, the Faeroe, Shetland, and Orkney Islands, the Hebrides, Norway north of Bergen, and Lapland) as well as the North American regions beyond the 50th parallel, including Newfoundland (a major part of which, however, lies south of that degree of latitude) and Greenland. Similarly cholera, though occasionally imported into South African ports, for example, in 1890 into Durban (Clemow), invariably failed to entrench itself, while the countries on the west coast of Africa south of Portuguese Guinea appear to have remained altogether free from the infection. In South America also cholera has remained absent from the southernmost parts of Chile and Argentina, and from the Falkland Islands. However, the appearance of the disease in the Archangel government situated on the White Sea in European Russia forms an interesting exception to this rule.

Besides the areas mentioned above, some islands such as St. Helena and Ascension, and the Bermudas, situated well away from continents, have remained exempt from cholera invasions.

It is no doubt true that cholera was far more frequent in areas situated north of the equator than in the southern hemisphere but, as shown by the frequency of violent manifestations of the infection in Indonesia and the repeated appearance of the disease south of the line in Africa and America, this unequal distribution cannot be due to factors of a strictly epidemiological nature.

An interesting question arising in this connexion is whether cholera ever gained an entry into the Pacific areas. As noted above, the claim of an inroad of the infection into western Australia deserves little, if any, credence. Lack of other pertinent information makes it also difficult to accept the statement of Simmons et al. (1944) that the disease was present during the nineteenth century in the Japanese Mandated Islands (Marianas or Ladrone Islands, the Caroline Islands, and the Marshall Islands), while the true nature of a few cases reported there in 1929-30 seems rather questionable. However, it deserves attention that, as asserted by Sticker, cholera was imported in 1893 into (German) New Guinea and continued to occur there without causing major havoc and that in 1896 the infection also gained a foothold in the Bismark Archipelago and the island of New Britain, areas situated comparatively near the frequently cholera-affected Indonesian archipelago.

Although fairly reliable figures are occasionally available, it is—as justly maintained by Haeser—altogether impossible to determine with even approximate accuracy the global mortality caused by cholera during the above-described pandemics. Nor is it possible to arrive indirectly at a reliable estimate by establishing in a generally valid manner the relation existing between the incidence of the disease, or the fatalities caused by
it, and the number of the inhabitants of the affected localities. This is impracticable not only because the percentage rate of cases and deaths was apt to show marked differences in different outbreaks, but also because quite often a panic flight of the people from cholera-stricken places led to a great reduction of the individuals actually at risk, while in other instances the presence of pilgrims or other non-residents resulted in a marked increase of the fuel available for the infection.

However, even though exact information is often lacking, there can be no doubt that, as asserted by Haeser, the loss in lives caused by cholera during the rather short course of its known history must be counted in millions. Great as this death toll must have been, it cannot compare in any way with the mortality caused in the past by plague, which is supposed to have killed 100 million people during the pandemic taking place in the sixth century and to have caused the death of 25 million in Europe alone at the time of the Black Death. It is, however, of great importance to note that, as indicated by the figures for India given in Table V, there is reason to assume that the number of fatalities caused by cholera is now greatly in excess of the death toll exacted by plague.

**TABLE V. DECENNIAL MORTALITY FROM CHOLERA AND PLAGUE IN INDIA, 1909-48**

<table>
<thead>
<tr>
<th>Decade</th>
<th>Cholera deaths</th>
<th>Plague deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>1909-18</td>
<td>247,068</td>
<td>422,153</td>
</tr>
<tr>
<td>1919-28</td>
<td>250,246</td>
<td>179,272</td>
</tr>
<tr>
<td>1929-38</td>
<td>189,190</td>
<td>42,288</td>
</tr>
<tr>
<td>1939-48</td>
<td>202,195</td>
<td>21,797</td>
</tr>
</tbody>
</table>

* After Swaroop & Pollitzer (1952) and Pollitzer (1954)

It must be admitted that the great reduction in the incidence of plague—evident not only in India but also in most other still-affected parts of the world—which set in long before it was possible to implement the improved methods for treatment and control now available, is due largely to intrinsic causes. There can be no doubt, however, that increasing use of these procedures is now bound to speed up the reduction of the disease. In the case of cholera, which, in India at least, has so far shown no signs of a really satisfactory decrease, methods of treatment and control combining easy application with full efficiency must still be sought. Hence, while in most respects the plague problem may be considered a res gesta, the many still-unsolved problems of cholera continue to call for urgent attention.
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Chapter 2

WORLD INCIDENCE *

GENERAL OBSERVATIONS

In the preceding chapter the history of cholera in the world has been traced from ancient times up to the year 1923. This period covers two main phases in the history of the disease, namely, that prior to 1817, during which cholera was confined to the East, if not almost exclusively to India, and a second period lasting from 1817 to about 1923, during which pandemics originating from India spread to countries lying east and west of India and, in some cases, swept over several continents of the world. During the following period, lasting from 1923 up to date, cholera became once more almost entirely a disease of the East, because during that time no major spread westwards beyond Afghanistan took place, with the exception of an invasion of Iran in 1939 and isolated epidemics which appeared in Egypt and in Syria in 1947-48, believed to have been due to extraordinary conditions created by the Second World War.

The appearance of the 1817 cholera pandemic at a time when British troops were occupying some important areas of India and were consequently severely affected by the disease, as well as the occurrence of a series of further pandemics causing world-wide havoc, have stimulated public health workers to record, in a continuous and gradually improved manner, the information bearing on the epidemiology of the disease. Historical facts relating to the incidence of cholera from year to year since 1817 have therefore been studied with considerable care and thus an increasing volume of valuable information, which now covers a period of almost seven score years, has become available. Based on these data a number of historical accounts, referred to in the first chapter, have been published as well as statistical studies on the prevalence and mode of spread of the disease, such as those of Rogers (1928) and Russell & Sundararajan (1928). Such studies, based as they were on an accumulating fund of statistical

* This chapter was written jointly with S. Swaroop, Ph.D., M.P.H., Chief Statistician, Health Statistical Methodology, World Health Organization, Geneva, Switzerland.
knowledge, have brought out certain well-established epidemiological patterns of the disease concerning its seasonal, geographical, and climatological variations, and its general mode of spread. In this chapter we propose first to comment briefly on some of these well-established facts concerning cholera incidence so that their knowledge may lead to a better understanding of the occurrence and spread of cholera in the world since 1923.

**Existence of Cholera-Endemic Foci**

As has been pointed out in the first chapter, each major epidemic spread of cholera since 1817 appears to have originated in the endemic home of the disease in India, where the infection has been entrenched since long before 1817, probably even since immemorial times.

Recent studies of the cholera mortality statistics, undertaken with the aim of demarcating the areas which truly harbour endemic foci of cholera in India, have drawn a distinction between:

1. Regions of large size where epidemics occur only occasionally, i.e., which remain free from infection for considerable periods, as for example the present States of the Punjab, Delhi, Madhya Pradesh, Hyderabad, Mysore, and Bombay.

2. Areas where the disease continues to be present at a fairly high level from year to year, and where it may at times assume epidemic proportions—examples are the State of Bengal, the coastal areas of Orissa, and certain districts of Bihar and Assam.

The areas of the second category are generally considered to be endemic to a varying degree, even though in some of the highly endemic areas of this kind it may not be possible, with the available laboratory techniques and facilities, to trace the spread of infection from patient to patient either directly or indirectly, particularly during the interepidemic periods.

On the basis of cholera mortality statistics relating to the period 1901-45, Swaroop & Pollitzer (1952) described the geographical distribution of the areas harbouring the endemic foci (see Fig. 1). The largest of these foci has its centre in Bengal (in both East and West Bengal now forming part of Pakistan and India respectively), in the deltaic region of the Ganges and the Brahmaputra, extending eastwards into Assam, westwards into Bihar, and possibly the eastern districts of Uttar Pradesh. Other endemic foci of lesser magnitude are found in the deltas formed by the Mahanadi in Orissa State, the Cauvery, the Kistna and the Godavari in Madras State, and possibly also in the Irrawady delta in Burma. Common factors to all these foci are that they are situated in close relation to surface-water systems, that they are densely populated areas at or near the coast, and that they lie at an altitude hardly exceeding 50 feet above sea level. There is reason to assume that, possibly owing to improvements in sanitation,
some of these foci have shrunk in size within recent years, so that presumably the endemic zones from which the infection could start its wide sway in the past were indeed extensive.

Because some of these foci, particularly those in and near Bengal, have been definitely known to exist since 1817, and also because in no other part of the world has cholera succeeded in establishing itself in such a permanent manner, the conclusion that the major endemic focus in Bengal has all along constituted the reservoir of the infection and the starting-point of the cholera pandemics, has often been reached, e.g., by Bryden (1874) and by Macnamara (1876). While the demarcation of the endemic foci in India supports this idea in general, any attempt to trace the origin of individual outbreaks to any single locality within this vast area would be futile. In fact, one must presume that epidemic outbursts in these regions lead to the production of a great volume of infection in different localities, sometimes even simultaneously at places distant one from another, and that under favourable climatic conditions the disease then spreads in a wave-like form to areas generally free from cholera.
Influence of Pilgrimage Centres and Festivals in the Spread of the Disease

India is a land of pilgrimages and shrines. Religious assemblies attracting thousands of devotees from various parts of the country take place at frequent intervals. In the past, the sanitary conditions at such congregations were appalling and, to make matters worse, almost invariably the ritual obliged the pilgrims to bathe in a common place in a river or tank. Under these circumstances, it is not surprising to find that such congregations often resulted in violent outbursts of cholera in non-endemic areas, the limit of spread of these epidemics depending to some extent upon the number and range of movement of the pilgrims, and upon favourable climatic factors. Characterizing the danger created by the pilgrimages, Rogers & Megaw (1952) stated:

"Each year about 20,000,000 pilgrims make long journeys in India to visit sacred, but often very insanitary shrines and they frequently disseminate the disease over large areas."

It is undeniable that these festivals, specially if held during seasons favourable to the propagation of the disease, play a most important role in the spread of cholera epidemics. At the same time, however, it has to be pointed out that the influence these gatherings exert in maintaining endemic foci in India is perhaps relatively unimportant. Swaroop & Raman (1951), studying the geographical location of such centres throughout the country, and the number of pilgrims they attract, reached the conclusion that "an explanation for endemicty of the disease must be sought elsewhere than in the occurrence of fairs and festivals".

Seasonal Factors Influencing the Spread of Cholera

Ever since statistical data on the incidence of cholera have been collected, it has been found that the disease shows a consistently similar and well-marked seasonal variation in individual parts of India, together with striking variations from area to area. It is of particular importance that almost each year the disease has shown in Bengal a tendency to become frequent during the period of September to November, and to reach a peak in December and January, followed by another seasonal increase in March-April (see Fig. 2). There is, however, considerable variation in the individual regions of Bengal.

Another important feature is that, as one proceeds westwards from Bengal, there is a marked tendency for the cholera peak to appear later, so that the winter months prove to be unfavourable to the spread of the infection in the contiguous States of Bihar and Uttar Pradesh (formerly
the United Provinces). Thus, as shown by Fig. 2, the seasonal cholera incidence reaches in these two States its peak in spring, while in the Punjab, the disease shows a relatively high incidence during the monsoon months of July and August.

**FIG. 2. MEAN MONTHLY CHOLERA DEATHS FOR FIVE INDIAN PROVINCES (1925-46)**

Fry (1925), studying this shift in the seasonal incidence of the disease as one proceeds westwards from the endemic home of cholera in Bengal, on the basis of the mortality figures for 1900-20, stated that

"It is in the Bhagulpur division between Rajshahi and Patna, that the change of seasonal incidence takes place. The curve for the whole division shows a transition stage, and when one comes to statistics of individual districts the change appears between the Purnea and Bhagalpur districts. The Purnea district is liable to severe spring cholera, and in Bhagalpur and further west the summer rainy season is the worst cholera time. This line is precisely that marked by the earlier writers as the western limit of the endemic area. It is noticeable that in this division not only does the seasonal incidence change, but the endemic cholera of Eastern Bengal changes to epidemic".

This phenomenon is of considerable importance in the progress of each major cholera pandemic, because epidemics originating in Bengal generally spread only as far as Bihar or Uttar Pradesh during the same year, subsiding during the cold season. Their westward progress was thus renewed only in the following year. This indeed was the sequence of events
in the pandemic commencing in 1817, when cholera, after a temporary quiescence during winter in Bengal and Bihar, became epidemic in the United Provinces (Uttar Pradesh) some time during the month of March 1818, and then spread from there in various directions. From 1923 onwards, the seasonal spread of cholera has followed the same “time-table” unless the occurrence of some fair or festival accelerated the progress of the disease.

The history of the spread of cholera from Bengal is usually, therefore, characterized by the rise of a seasonal wave in the endemic home, followed by a recrudescence in Uttar Pradesh, from where the disease is carried northwards through the Punjab along the land route to Afghanistan, Iran, etc. Each year, the incidence of cholera in the Punjab subsides during the winter months, but it is of interest to note that during these months a spread of the infection into Kashmir was apt to take place, as exemplified by the description of the 1925-26 epidemic in that State.

The spread of cholera from Bengal through the United Provinces and the Punjab north-westwards through Afghanistan has followed what Bryden (1874) called the “northern epidemic highway of the disease”. This author also described a “southern epidemic highway”, along which cholera spread from the United Provinces through central India southwards to the States of Madras and Bombay, sometimes reaching Ceylon, and being carried by sea from Bombay to the Western world. Ever since records have been available, the disease in its westward spread from India has continued to follow these general routes.

A surprising feature of the spread from Bengal is that cholera has never reached southern India by what is the shortest and direct route geographically through Orissa along the east coast, the more so as an important endemic focus of the disease is located at the delta of the Mahanadi River. The explanation offered in 1871 by Cornish (quoted by Rogers, 1928) was “that the disease was checked by the sparsely inhabited hill tracts reaching down close to the coast at this point.”

The history of the spread of cholera from India to countries eastwards is a record of maritime transmission, seaports in that part of the world being liable to primary outbreaks followed by progress of the infection to the hinterland. Small coastal craft have undoubtedly played a part in the local diffusion of the infection.

Disappearance of Cholera from the West

The figures of Table VI, showing the mortality due to cholera throughout the world from 1900 to 1954, well bear out the statement made at the beginning of this review that, apart from some occasional inroads taking place as a rule under extraordinary circumstances, from 1923 onwards the manifestations of the disease remained virtually restricted to India and some countries to the east of it.
FIG. 3. DISTRIBUTION OF CHOLERA IN THE WORLD, 1800-1957
Incidence in 1957 and Dates of Last Occurrence, 1800-1956

Main map: Distribution of cholera in 1957 based on provisional notification of cases
A = 1 case  B = 2-25 cases  C = less than 4 cases per 100,000 population  D = 6-10 cases per 100,000 population  E = 12-25 cases per 100,000 population  F = 26-50 cases per 100,000 population  G = date of last occurrence during the period 1946-56

Inset map:
= date of last occurrence during period 1800-1945  II = areas affected during the period 1879-1911

1946-1957

EGYPT

1800-1945

Main map: Distribution of cholera in 1957 based on provisional notification of cases
A = 1 case  B = 2-25 cases  C = less than 4 cases per 100,000 population  D = 6-10 cases per 100,000 population  E = 12-25 cases per 100,000 population  F = 26-50 cases per 100,000 population  G = date of last occurrence during the period 1946-56

Inset map:
= date of last occurrence during period 1800-1945  II = areas affected during the period 1879-1911
It is curious to note that, besides marking the end of the last cholera pandemic, the year 1923 is also remarkable in so far as the cholera mortality then reported in India, though still considerable, was the lowest on record for any year since the beginning of the century. In fact, during two months of 1923 not a single cholera death was reported from an area comprising about half of India, including Uttar Pradesh and States situated on its west and south-west. Seeing, however, that (a) cholera again became far more rampant in India in some of the subsequent years without spreading westwards, and (b) it had disappeared from most Western countries well before 1923, not much significance can be ascribed to the low incidence of the disease in India during that year. The true, rather complex, causes of the disappearance of cholera from the west will be discussed later.

RECENT INCIDENCE OF CHOLERA

Cholera in Russia

The figures in Table VII, showing the case incidence of cholera in Russia from 1823 to 1926, though incomplete as far as the period prior to 1904 is concerned, serve to indicate the relative magnitude of the manifestations of the disease during the various invasions of the country. It will be gathered that, following the peak year of 1921, during which the situation became almost as serious as in 1910, cholera was still quite frequent in 1922, but that then a rapid decline set in.

Of the 114 cases reported during 1923, 73 occurred during an outbreak in the city of Rostov-on-the-Don, the remainder being sporadic occurrences in widely separated localities. A detailed account of the history of cholera in Rostov-on-the-Don from 1920 to 1925 was published by Barikine & Cazenave (1925).

After the disappearance of cholera from Russia in 1926, no outbreak of the disease has occurred in any European country with the possible exception of a small and rather doubtful epidemic during the Second World War in the German Army in the Ukraine. According to Stowman (1945):

"A statement was made by Dr. Heilmeyer of Jena at the meeting of the Deutsche Gesellschaft für Innere Medizin at Vienna, 10-14 October 1943, to the effect that cholera had been no problem to the Germans during the present war, because the only outbreak which had occurred had been one of 78 severe cases in the German Army in the Ukraine [no date given]. The word 'cholera' was not qualified, and it is barely possible that he meant cholera nostras, which, however, is a rather vague term, usually covering outbreaks of food poisoning due to Salmonella or Proteus infection.

1This review is based mainly on a study of the final figures of cholera incidence available up to the end of the year 1954. However, in order to illustrate the most recent trend of the infection, a map has been included showing cholera distribution in the world during the period 1800-1957 and the incidence of cholera in 1957 (Fig. 3).
<table>
<thead>
<tr>
<th>Years</th>
<th>India &amp; Pakistan</th>
<th>Burma</th>
<th>Ceylon</th>
<th>Thailand</th>
<th>India-China</th>
<th>Java</th>
<th>China</th>
<th>Shanghai</th>
<th>Hong Kong</th>
<th>Korea</th>
<th>Malaya</th>
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<td>86,668</td>
<td>3,491</td>
<td>-</td>
<td>-</td>
<td>E</td>
<td>-</td>
<td>-</td>
<td>7,103</td>
<td>-</td>
<td>15</td>
<td>3</td>
<td>2</td>
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<tr>
<td>1911</td>
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<td>1,191</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>1921</td>
<td>1,151</td>
<td>3,690</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>12</td>
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<tr>
<td>1941</td>
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<td>3,590</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
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</tbody>
</table>

E = Cholera prevalent in epidemic form.
C = Local outbreaks or sporadic cases.

a Former British provinces
b Excluding figures for East Bengal
c Provisional figures
## World Incidence

IN VARIOUS COUNTRIES FROM 1900 TO 1954

<table>
<thead>
<tr>
<th>Years</th>
<th>Taiwan</th>
<th>Japan</th>
<th>Philippines</th>
<th>USSR</th>
<th>Egypt</th>
<th>Iran</th>
<th>Iraq</th>
<th>Syria</th>
<th>Austria</th>
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<th>Hungary</th>
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<td>613</td>
<td>8,164</td>
<td>9302</td>
<td>1,993</td>
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<td>85</td>
<td>656</td>
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<td>1,880</td>
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<td>2,177</td>
<td>861</td>
<td>820</td>
<td>864</td>
<td>22</td>
<td>33</td>
<td>1,604</td>
<td>2,650</td>
<td>3,992</td>
<td>228</td>
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<td>41</td>
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<td>4,397</td>
<td>955</td>
<td>389</td>
<td>53</td>
<td>27</td>
<td>36</td>
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<td>1</td>
<td>3</td>
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<td>1</td>
<td>1</td>
<td>1</td>
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<td>1,147</td>
<td>1,147</td>
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<td>1</td>
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</table>

* Figures not available.
--- No cholera death recorded.
### Table VII. Cholera in European Russia, 1823-1926

<table>
<thead>
<tr>
<th>Year</th>
<th>Cases</th>
<th>Year</th>
<th>Cases</th>
<th>Year</th>
<th>Cases</th>
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<td>1850</td>
<td>4931</td>
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<td>1829</td>
<td>3590</td>
<td>1860</td>
<td>isolated cases</td>
<td>1908</td>
<td>30705</td>
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<td>1866</td>
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<td>3416</td>
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<tr>
<td>1833</td>
<td>14428</td>
<td>1867</td>
<td>6245</td>
<td>1912</td>
<td>9</td>
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<tr>
<td>1834</td>
<td>isolated cases</td>
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<td>310</td>
<td>1913</td>
<td>324</td>
</tr>
<tr>
<td>1837</td>
<td>isolated cases</td>
<td>1869</td>
<td>1275</td>
<td>1914</td>
<td>9715</td>
</tr>
<tr>
<td>1838</td>
<td>isolated cases</td>
<td>1870</td>
<td>21564</td>
<td>1915</td>
<td>46455</td>
</tr>
<tr>
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<td>180846</td>
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<td>322711</td>
<td>1916</td>
<td>1800</td>
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<tr>
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<td>1742439</td>
<td>1872</td>
<td>310507</td>
<td>1917</td>
<td>130</td>
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<tr>
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<td>1873</td>
<td>9943</td>
<td>1918</td>
<td>41586</td>
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<tr>
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<td>54</td>
<td>1874</td>
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<td>1919</td>
<td>5119</td>
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<td>1920</td>
<td>29615</td>
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<tr>
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<td>1877</td>
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<td>86173</td>
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<tr>
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<td>331025</td>
<td>1878</td>
<td>46</td>
<td>1923</td>
<td>114</td>
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<td>2167</td>
<td>1924</td>
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</tr>
<tr>
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<td>1811</td>
<td>1880</td>
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<tr>
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<td>3649</td>
<td>1881</td>
<td>598</td>
<td>1926</td>
<td>1</td>
</tr>
</tbody>
</table>

"The clinical description, however, seems to tally with that of Asiatic cholera, and so does the treatment—intravenous injections of saline solutions. The fatal cases, the proportion of which is not given, but appears to have been considerable, showed pronounced uraemia. It was proved that the disease was not waterborne. Bacteriological and serological examinations showed atypical milder cases, some of them ambulatory, among the contacts. This may occur also in epidemics of Asiatic cholera.

"It will be recalled that following the epidemic of Asiatic cholera in the Ukraine 1918-1922, choleriform vibrios and phosphorescent vibrios were still found in 1925 in the waters of the Don at Rostov and in fish and shrimps taken in these waters. It was found that ingestion of these vibrios caused an acute cholera attack and that typical cholera vibrios appeared in the stools. The case mortality rate of such infections was lower than in classical cholera. Abortive cases and carriers were found. At any rate, the infection can hardly be called true Asiatic cholera".

### Cholera in Non-European Countries West of India

**Iraq**

Among Asiatic countries lying west of India only Iraq and Iran recorded high figures during 1923.
The epidemic in Iraq, causing 1503 cases and 1110 deaths, began in August 1923, reached its peak in September (773 cases, 585 deaths), and disappeared completely from the country by the end of the same year. The most seriously affected city was Basra, with 585 cases and 438 deaths. After the 1923 epidemic, two more outbreaks occurred in Iraq, one in 1927 (1063 deaths) and the other in 1931, which also began in Basra. The cause of the outbreak commencing in July 1931 was the disembarkment of two cholera patients coming from the infected port of Bombay. An epidemic immediately developed at Basra and lasted until the middle of November. The infection also spread along the Tigris and the Euphrates, the total mortality in Iraq amounting to 1548. Since 1932, that country has remained free from cholera.

Iran

In Iran, the infection was present in a mild form during 1922, when 28 cases and two deaths were recorded. The epidemic outbreak of 1923, causing 1029 deaths, was mostly confined to the ports of Abadan (992 deaths) and Mohammerah (27 deaths), and in its spread was a continuation of the epidemic that had had its origin in Basra, Iraq. An extension took place along the Karun River as far north as Khurramabad. Three more epidemic outbreaks have occurred in Iran during the period under review, namely during 1927 (593 deaths), 1931 (165 deaths), and 1938-39 (307 deaths). During 1938, the incidence of cholera was relatively high in the Punjab, India, from where the disease spread through Afghanistan to Iran. The 1939 outbreak in Iran is known to have lasted only two months and came to an end early in August. From 1940 onwards Iran has remained free from cholera.

Afghanistan

Cholera appeared in north India in several districts near the Afghan border in May 1930 and continued till October of the same year. In Afghanistan, an epidemic broke out in July 1930 in the valley of the Kabul River and affected the major towns of Kabul, Jellalabad and Charikar. Further south the disease spread in July to Ghazni (where over 160 cases were reported in two days), reaching Kandahar and Makur Kalat in August. In the latter towns it is known to have prevailed with "marked severity" and to have abated in September 1930. The total number of cases and deaths is not known.

After six years another outbreak of cholera was reported in Afghanistan during 1936. According to the 1938 report of the Public Health Commissioner with the Government of India, the disease was also present in 1937. Becoming once more epidemic, cholera claimed 2141 victims in 1938, while during the following year 849 deaths from this disease were recorded in the country. As stated by Biraud & Kaul (1947) "very small outbreaks" of cholera took place in Afghanistan in 1941 and in 1946 (35 cases).
Egypt

In the post-war years the Western countries remained free from cholera until 1947, when a minor outbreak lasting until 1948 commenced in Syria, and a severe epidemic spread in Egypt.

Cholera had been absent from Egypt since 1919 when it made a sudden appearance on 22 September 1947. While previous outbreaks in that country had usually been due to an importation of the infection through pilgrims returning from Mecca, this did not hold true of the 1947 epidemic, which broke out before the pilgrims had returned, and nearly a month before the great festival of 20 October.

The outbreak began at El Korein, an important trading centre of some 15,000 inhabitants on the eastern fringe of the Nile delta, situated close to the canal which provides drinking water for the cities and villages along the Suez Canal, at a time when merchants had congregated from all provinces to attend the annual date fair.

In addition, some 6000 workmen, engaged in construction work near by, were at the time billeted in El Korein village, and it is believed that the panic flight of this floating population immediately after the appearance of the disease and before local quarantine could be enforced accounted for the rapid diffusion of the infection. In three days the presence of cholera was noted at Cairo, on 27 September it had reached Ismailia, and by the 29th the whole of the Kalyubiya and Dakahlia Provinces as well as Sharkiya were affected, besides the Suez area. In three weeks all provinces of Lower Egypt were involved, and by October 1947 the provinces of Upper Egypt had been reached by the infection. However, by December cholera had practically disappeared from Egypt.

While it was impossible to ascertain how the outbreak originated, the Egyptian health authorities were of the opinion that the infection had in all probability been imported from India. In this connexion, the following extract from the Lancet (1947) may be quoted:

"The source of the epidemic is still obscure and no official statement concerning its discovery has been made in Egypt. But many first-hand observers think they can trace the origin of the infection to Egyptian labourers infected by aeroplanes coming from India to British Army aerodromes, where these labourers work. It seems that some of the first cases were actually engaged in these airfields, which—until 6 October—were not under the control of the Egyptian Quarantine Department. There is also a coincidence between the Egyptian epidemic and that which started on 15 August 1947 in the Punjab, after its partition between Pakistan and Hindustan, and the migration of about 5,000,000 persons which followed it. In support of this view they point to the return from India of British troops, who used the Suez Canal region as a quarantine station and remained there with their Indian retinue for 2 weeks before departing for England. British authorities, however, deny that there is any relation between the present epidemic and their Forces. It has certainly been established that the epidemic is in no way related to pilgrimage."

An editorial in the British Medical Journal (1951) contained the following statement:
"The source of the infection still remains a mystery for, practically at the same time, cholera was discovered over a wide area between Cairo and Abou Sueir, a distance of 132 kilometres. Although cholera was prevalent at the time in Pakistan and many British service-men were transferred from India to Egypt by air, cholera did not break out in the Canal Zone, but in areas where no British were stationed."

Lasting for a period of about three months, the epidemic led to 32,978 cases, with 20,472 deaths. Exhaustive accounts of the manner of its spread, its epidemiology, and the control measures adopted have been given by Shousha (1948), Khalil (1948), and by Biraud & Kaul (1947).

It is of interest to note that, while, in the past, Egypt used to serve as a stepping-stone for the spread of cholera from Asia to Europe, the 1947 epidemic did not lead to a westward spread of the infection either by ship or by aircraft. On the other hand, while there is no statistical evidence to suggest that cholera has been or is endemic in Egypt, the 1947 outbreak proved that, once the infection has been imported, its spread is facilitated by the environmental conditions prevailing in the rural areas of the country.

As stated by Stowman (1945):

"The powerful sanitary barrier set up in the Red Sea and at the Suez Canal has functioned to full satisfaction as far as cholera is concerned. Not a single cholera case has come through to Europe that way for 30 years."

**Syria**

A cholera outbreak of unknown origin commenced in Syria on 20 December 1947, i.e., after the disease had almost disappeared from Egypt. Seven confirmed cases were first reported from two neighbouring villages in the Hauran Province, the infection later spreading to three more villages in the same province. All five villages were on the main Dera-Damascus road. Altogether 45 cases and 18 deaths were reported (Lancet, 1948). Three non-fatal cholera cases occurred in Syria in 1948.

**Arabia**

Prior to the period 1923-53, cholera used to be the scourge of pilgrims journeying to the Hejaz. Duguet (1931) stated that there had been 27 epidemics during the Mecca pilgrimage in the previous 81 years and that the Hejaz was indeed a relay station of cholera in its progress from the east towards the west. At times, the cholera mortality in Mecca assumed grievous proportions, the death-roll at the peak of the 1902 outbreak, for instance, amounting to 800 to 1000 daily. During the 1907-08 pilgrimage more than 20,000 cholera deaths occurred.

The last epidemic in Mecca took place during the years 1910-12. Since 1913, the pilgrimages have remained free from cholera, with the possible exception of one which took place in 1930, and which was considered "infected" although no case or death from cholera was reported in the Hejaz because in May a pilgrim returning from Mecca was landed at Massawa, Eritrea, with symptoms of the disease.
Cholera in Insular Countries of Asia

Philippines

The insular countries of the Far East have also remained fairly free from cholera in recent years. For instance, Table VI shows that since 1923 in the Philippines there have been only two epidemic waves, one reaching its peak in 1925, the second outbreak starting in the year 1930 and continuing for five successive years, i.e., until 1934, during which period the disease prevailed in the central, densely populated archipelago. In November 1933, the disease was present on the west coast of Bohol Island and the opposite east coast of Cebu Island. While it disappeared from the latter island early in 1934, it spread to the east coast of Negros Island, but died out by April 1934.

From 1938 onwards the islands have remained free from cholera.

Indonesia

The island of Java recorded its last major epidemic of cholera in 1918-19. Thereafter, as shown in Table VI, only sporadic cases have occurred. Thus, in the year 1927 there were 10 deaths, and in 1928 only one death from the disease. The island has now been free from cholera for over a quarter of a century. Even in earlier years following the First World War, the infection, when imported, was quickly exterminated.

An outbreak which aroused considerable epidemiological interest started in September 1937 on the island of Celebes, Indonesia. The first cases were notified from Pangkadjene, which is situated about 40 miles north of Macassar. The patients in question showed clinical symptoms of cholera, and the vibrios, isolated from the stools of some of them, were shown to be of the El Tor variety. From 26 September to 11 December 1937 there were 17 cases and 11 deaths in this area. The disease did not manifest itself again until 19 January 1938, when there were 12 deaths up to 19 February. The total number of cases from 26 September to 29 April 1938 was 35, 27 of whom died. It was reported that from 31 August 1937 to 23 September 1937 already six people had died with symptoms suggesting cholera infection on the island of Samtollë north-west of Macassar. Three further cases occurred on the same island towards the end of September, two of whom were fatal. From these patients also El Tor vibrios were isolated. The epidemiological features of this outbreak and the characteristics of the vibrio isolated have been discussed by de Moor (1938).

A further instance of infection with the El Tor vibrio was observed in Celebes on 27 October 1939, the patient concerned dying within 24 hours after the appearance of symptoms. To quote from the annual report for 1939 of the League of Nations Singapore Bureau:

"In the area in which this first case occurred there were 20 further cases in the succeeding two months while further south 5 cases occurred in one week in a small
kampong containing 2,000 inhabitants. All the cases had symptoms which clinically resembled cholera and the fatal cases died within 12 to 24 hours of their onset. The mortality rate appears to reach 50%-60%, but there may be a number of unrecognized mild cases.

"A point of some interest is the finding of carriers in a percentage of 10%-15% among contacts of cases and a percentage of 0.5% in infected kampongs. Of special importance was the finding in one kampong, which as far as could be ascertained was quite free from infection, of 15 carriers among 1,000 persons examined—a percentage of 1.5%.

"As far as could be ascertained, the first patient has not been near any areas in which infection occurred in 1937/1938, nor could any people be found in her neighbourhood who had been in these districts; in other words no evidence whatever could be found to indicate contact with any of the areas where infection was known to have previously occurred.

"Following the discovery of the first case, 2 others were found. The second patient had vomiting and diarrhoea with fever but was well again in 4 days, while the third patient remained ill for several days."

The following remarks are quoted from the annual report for 1940 of the League of Nations Singapore Bureau.

"During the period 16 June to 27 July 1940 a further 8 cases of El Tor infection were notified from Celebes, of which 5 were fatal.

"Some interesting points in regard to this disease have been brought to notice. One of these is the high mortality, viz. 65% which resembles that of true cholera. Another is that it does not tend to assume epidemic proportions; for example, there was only 1 case in each of 14 villages, 2 cases in each of four villages and 5 cases in one village only. In addition, with odd exceptions, there was never more than 1 case in a family."

It is relevant to state that in India the El Tor vibrio has repeatedly been found in water, even in areas entirely free from cholera. The question whether this vibrio may be etiologically related in India to any choleraic disease, as was evidently the case in Celebes, has not yet been satisfactorily answered.

Singapore

Singapore Island has been free from the disease since the last outbreak in 1926-28 (see Table VI).

Japan

Table VI shows that prior to the Second World War the disease had almost completely disappeared from the Japanese islands, although early in the present century comparatively severe epidemics were not infrequent. These manifestations were held to have been invariably due to an importation of the infection. Generally, cholera gained impetus in the large seaports and was then spread along the coast by small craft, whose movements were difficult to control. According to Takano and co-authors (1926) the disease

"is most frequently imported in the months of August and September and these are the months in which epidemics reach their peaks. The epidemic begins to subside gradually in October and November and practically ceases in December."

During May 1946, however, the arrival of repatriation ships from China and other countries of the Far East led to an importation of cholera
through the port of Uraga. The epidemic soon spread and the incidence of the disease during the year reached a total of 1229 cases with 528 deaths. From 1947 onwards Japan has been completely free from cholera.

Taiwan

Although no complete figures for Taiwan are available, Table VI indicates an almost negligible incidence of the disease in the island from 1923 to 1943. Even the major epidemic taking place on the Chinese mainland in 1932 caused only minor repercussions in Taiwan. For, as reported by Shimoji and co-authors (1933), the importation of the infection in 1932 by the crew of a ship led to the appearance of only 17 manifest cases (with six deaths) and to a carrier state in six further apparently healthy persons. Twenty out of these 23 infected individuals had attended the funeral of the captain of the junk and had consumed obviously cholera-contaminated food with the crew.

However, serious cholera outbreaks, due to an importation of the infection, took place in Taiwan in 1943 and in 1946.

Ceylon

The history of cholera in Ceylon is that of repeated importations of the infection from India. As shown by a comparison of the series of annual figures for the two countries embodied in Table VI, outbreaks in Ceylon occurred generally one year after a high incidence of the disease had been recorded in India.

The last major outbreak, resulting in 419 deaths, took place in Ceylon in 1919 when cholera showed a peak incidence in India. From 1947 onwards the incidence of the disease in Ceylon has been negligible.

Cholera in the Asiatic Mainland

World cholera mortality rates per 100,000 population in the recent five-year period 1950-54 are shown in Table VIII. Because of their size, the figures for India and Pakistan are shown separately by individual States.

The total deaths from cholera during 1950-54 constituted only about 0.1% of the estimated deaths from all causes in the world.

Fig. 4, showing the geographical distribution of the disease, indicates that in the recent post-war years cholera has been confined mainly to the Asiatic mainland, largely to India, East Pakistan, and Burma. The percentages of the world cholera deaths reported from these countries are:

<table>
<thead>
<tr>
<th>Country</th>
<th>%</th>
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</thead>
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<td>India</td>
<td>75.48</td>
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<tr>
<td>Pakistan</td>
<td>22.57</td>
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<td>Burma</td>
<td>1.93</td>
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99.98
<table>
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<tr>
<th>Country</th>
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<th>Country</th>
<th>Cholera death-rate</th>
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<tr>
<td></td>
<td>mean</td>
<td>median</td>
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</tr>
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<td>—</td>
<td>23.4</td>
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<td>3.0</td>
<td>11.6</td>
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<td>34.8</td>
<td>31.5</td>
</tr>
<tr>
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<td>4.8</td>
<td>11.4</td>
</tr>
<tr>
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<td>—</td>
<td>41.9</td>
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<td>—</td>
<td>7.9</td>
</tr>
<tr>
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<td>37.3</td>
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<td>3.3</td>
</tr>
<tr>
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<td>27.7</td>
<td>3.3</td>
</tr>
<tr>
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<td>14.3</td>
<td>3.3</td>
</tr>
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<td>Punjab</td>
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<td>0.1</td>
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</table>

This shows that India and Pakistan together constituted 98%, and with Burma 99.98%, of the total cholera deaths in the world during 1950-54. Within India and Pakistan the highest incidence of the disease is reported from its endemic home in the two Bengals (East and West), into which the

**FIG. 4. GEOGRAPHICAL DISTRIBUTION OF CHOLERA, 1950-54**

Median death-rate per 100,000 inhabitants (1948-54)

- less than 0.01
- 0.01-0.9
- 1.0-4.9
- 5.0-10.9
- 20.0 and over
old presidency of Bengal was divided following the partition of the country in August 1947.

An account of the recent occurrence of cholera in these and the other countries involved on the Asiatic mainland follows.

China

As summarized by Wu Lien-teh and co-authors (1934), 46 cholera manifestations of a more or less serious nature were recorded in China from 1817 to 1934, ten of which led to a spread of the infection as far north as Manchuria. Following a serious and a widely disseminated outbreak in 1919, during the period under review cholera epidemics in China were recorded in 1926, 1932, 1937-39, 1942, and 1946. From 1937 onwards, owing to war conditions, the disease showed a tendency to become more widespread and persistent than had been the case in the past. However, as will be shown later, the period after 1948 is characterized by a most marked decrease of cholera in China and evidently its ultimate disappearance.

Reliable numerical information regarding the incidence of cholera in earlier years in China is not available because of the insufficient system of disease-reporting. It was only in 1926 that the National Epidemic Prevention Bureau at Peking inaugurated a system for the recording on the epidemic situation. Nevertheless, the large size of the country, the inadequacy of health staffs, and civil wars rendered it impossible to obtain complete records. The information available, therefore, indicates only the relative magnitude of the cholera prevalence from year to year and its seasonal variation. But no proper estimate of the total incidence can be made.

It has been shown by Swaroop & Pollitzer (1952) that during 1939-48 the area where cholera showed the greatest tendency to persistence lay in the delta of the Si-kiang River in the two southern provinces of Kwangtung and Kwang-si, possibly extending from the coastal and deltaic regions of Kwang-tung westwards to Yun-nan and northwards along the coast into Fu-kien Province. Some persistence of the infection to a comparatively minor degree has also been observed in Hu-nan in a locality on the Yuan River near Tung-ting Lake. The deltaic areas of the Yangtze river do not seem to have been as favourable for the persistence of the infection as the Si-kiang Delta.

Ever since statistical data were collected for certain provinces of China, no year, until 1947, was found free from the disease, which was thus constantly present in some part of the country. Still, the absence of a really prolonged persistence of the infection in any of these areas renders it likely that most of the major epidemics in China were due to importations of the infection, if not from abroad, at least from areas within the country, where a state of what might be called "temporary endemicity " had established itself. It has to be mentioned, however, that Stowman (1946) was not in agreement with this assumption, maintaining that "there can be little
doubt that cholera has now become endemic in several parts of China.” It is noteworthy, however, that he qualified this statement by adding that “nevertheless, there is evidence to show that it is not endemic in many areas which have been visited by severe epidemics.”

The numbers of cholera cases reported in the various provinces of China from 1939 to 1950 are shown in Table IX.

Exact information regarding the number of cholera cases or deaths for an earlier period is not available, but an approximate idea of the incidence of the disease has been provided in Table VI.

During the years 1949-50 the mainland of China appears to have been free from cholera, and, to judge from press reports becoming available in March 1957, the infection has continued to be absent. This state of affairs supports the contention that cholera is not endemic in China.

The first epidemic occurring during 1923-54 arose in China in 1926. It is known that even during 1925 cholera was widespread in the country, only Yun-nan in the extreme south-west having remained entirely free from the infection. Nevertheless, during that year the disease was largely confined to the coastal plains north and south of the Yangtze as well as to the whole of the south coast. Having been prevalent in 1925 during the months of July and August, cholera appeared in mild form in the Provinces of Che-kiang, An-hwei, Honan, and Shan-si in the earlier part of 1926. In the months of July and August 1926, however, epidemics involving all the coastal districts up to Manchuria appeared, while in Shanghai the infection of the Chapei waterworks led to a serious situation. The disease also spread to Korea in 1926, where 13 districts were reported to have become affected. However, owing to strict measures, each of these manifestations was quickly controlled so that only 159 deaths resulted.

In 1927, the disease was only moderately prevalent in Shanghai but there were serious epidemics in Kwang-tung Province in the south and Chihli Province in the north. During 1927 to 1931, although several areas continued to report cases, the incidence was much lower except that Shanghai had an epidemic in July 1929 which, however, was less severe than that of 1926.

In 1932, China suffered from a severe manifestation of cholera which could well be regarded as pandemic in character, outbreaks occurring in all the eastern provinces in the north as well as the south. A rough idea of the incidence of these epidemics and the extent of their spread is provided by the figures in Table X.

During 1931, in spite of disastrous floods in the Yangtze valley, the cholera incidence in China remained very low, only one death being recorded in Canton while the ports and provinces in the north remained free from the infection. In March 1932, the disease first appeared in epidemic form at Canton, then in April at Shanghai. During May, cholera spread to Swatow and inland to Han-kow and Nanking, and by June it had also affected several
TABLE IX. CHOLERA CASES REPORTED IN CHINA FROM 1939 TO 1950

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<tr>
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* In thousands
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</tr>
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<td>Total</td>
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<td>100666</td>
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</tr>
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</table>

* From the Report of National Flood Relief Commission 1931-2, quoted by Wu Lien-teh and co-authors (1934).

Manchurian ports in the north (Antung, Newchwang, and Dairen). In July, the infection had reached the north-western provinces of Sui-yuan, Shen-si and Hu-nan. The floods in Manchuria in August aggravated the epidemic situation. The number of cases was estimated at more than 100,000 and that of deaths at 31,974. Although cholera-infected boats are known to have carried the infection to Japan, the general effect of the great epidemic in the countries outside China was that it appeared only in the form of short outbreaks with comparatively few victims.

Cholera in China subsided during the year of 1933, when only a very low incidence was recorded. Except a few isolated cases in the ports of Canton,
Shanghai, and Han-kow, the year 1934 also remained almost free from the disease.

In May 1937, cholera once more broke out in epidemic form in the eastern part of Kwang-tung, and by August had reached Kwang-si. It is estimated that over 28,000 cases occurred in the former province but only 600 in the latter. By December of 1937 the infection became manifest in the Yuan River basin and in June of the following year three other river systems, namely those of the Tse, the Siang, and the Yangtze, were found to have become involved. The epidemic had thus spread as far north as Hu-nan where over 4,500 cases with more than 2,000 deaths occurred.

In 1939, another serious outbreak took place in the Sze-Chwan and Shen-si Provinces, where the two major cities of Chung-king and Cheng-tu became distributing centres of the infection. This outbreak is believed to have originated towards the end of June in the hilly rural regions of Nangpu Hsien (district), situated on a tributary of the Kialing River, from where it spread to a number of neighbouring districts to the west and the northwest, causing over 41,000 deaths in Sze-Chwan and more than 10,000 in Shen-si. While only 470 cases were reported in Hu-nan to the south, Hu-peh and Kiang-si Provinces became seriously involved. Besides thus reaching its highest incidence in central China, cholera also spread into south-western China. However, by the end of August the epidemic had practically subsided.

The low incidence in the interior of China during 1941 is of interest in view of the fact that cholera was rampant in that year in the port cities of Macao (1,475 cases), Hong Kong, Canton, and Shanghai. This led Stowman (1945) to observe that “it is clear... that cholera epidemics in the interior do not necessarily follow upon high prevalence in the ports”.

The 1942 epidemic was mainly confined to south China, where the infection continued to exist, leading to an outbreak in Kwang-tung in September 1945 and to a recrudescence of this epidemic in May of 1946. The appearance of cholera in Hu-nan in 1945 probably stood in causal connexion with its presence in Kwang-tung.

During 1945, the disease was also present in mild form in Kwang-si, Kwei-chow, and Yun-nan. The extensive outbreak taking place in that year in Sze-Chwan started in May on the Yangtze above Chung-king. This city and its neighbourhood became affected early in June. An extension of the infection down the Yangtze River as far as Ichang followed and, while the epidemic in Sze-Chwan had subsided by the end of 1945, cholera continued to exist with moderate intensity at Ichang throughout the winter. This presence of the disease at Ichang seems to have been responsible for a severe outbreak among the Japanese concentrated in a camp at Han-kow during February 1946. Being afterwards transported in barges from Han-kow, the former inmates of this camp carried the infection to Nan-king and Shanghai.
Even though August was usually the month of highest cholera incidence, the generally mild but most extensive outbreak taking place in China during 1946 gained momentum and started to spread in July. By October 1946, the disease had reached as far north as central Manchuria and Inner Mongolia, as far south as Hoppo (Lim-chow) east of the Tong-king border, as well as 100 miles west of Chung-king.

Thus, while the epidemics of 1926, 1932, and 1942 had affected chiefly the lower and middle Yangtze areas, and the 1937-39 outbreaks had been most severe farther west, the 1946 epidemics extended to the utmost confines of the country. There can be no doubt that this exceedingly wide spread was due to the return of displaced persons as well as to troop movements and the repatriation of Japanese internees.

The ravages of the 1946 outbreaks were worst, by far, in Manchuria, where the disease had been absent for many years. Probably imported by troops coming from south China, cholera appeared in Manchuria in the second half of June at Liao-ning. An explosive spread of the infection followed, and the area outside the Communist zone was affected within 2-3 weeks, the infection mainly spreading along the railway lines. Though, generally, information is available only for cities and communities along the railway lines, there can be no doubt that in 1946 cholera exacted a death-toll in Manchuria which equalled or even surpassed that caused in this part of China by the previous most severe visitations of the disease.

Though there were probably also some smaller foci where the infection had persisted during the winter of 1945-46 to become recrudescent in spring, it appears that there were two main foci from where the 1946 epidemic originated—the one in Kwang-tung, the other on the middle Yangtze. The outbreaks traceable to these main foci, though becoming to some extent superimposed, remained distinct in their behaviour. The Yangtze epidemics were characterized by a low or moderate case fatality rate (approximately 10%), while in case of the outbreaks derived from Kwang-tung the case fatality was as high as 25% to 30%.

**Thailand**

The trend of cholera in Thailand from 1917 to 1954 is illustrated in Fig. 5. During the last few years this disease has been almost absent from the country, for while in 1948 it still caused 15 deaths, only one cholera fatality was recorded in 1949 and none during the years 1950-54. A solitary cholera case was observed in the Province of Prachinbury in 1951.

In marked contrast to India, East Pakistan, and Burma with a persistently high cholera incidence, Thailand presents features characteristic of an area liable only to epidemic inroads of the infection. For, as shown in Table VI, epidemic periods with a high cholera mortality, such as those of 1925-29, 1935-37, and 1943-47, lasting 3-5 years, alternated with interepidemic
Although cholera is apparently not endemic in Thailand, its importation has led in the past to violent and prolonged epidemics. For instance, the arrival in Bangkok, in October 1925, of a cholera-stricken steamer from Swatow led to an outbreak lasting from November to December in the former port, which caused over 3000 cases with more than 2000 deaths. After a temporary seasonal decrease in January and February 1926, the infection flared up once more and, leading to a peak incidence in May, continued to be manifest until the end of October. This epidemic reached French Indochina through Cambodia in January 1926.

The 1935 epidemic was presumably imported from Burma.

The last epidemic, which terminated in 1947, was also of prolonged duration, being spread over a period of five years. River and canal water-pollution is believed to be the greatest source of danger in the dissemination of cholera in Thailand, the disease being prevalent for longer periods in the southern provinces along the river Chao Phya.

**Indochina (Cambodia, Laos, Viet Nam)**

Like Thailand, Indochina is an area liable to cholera epidemics of a highly explosive nature.

A comparison of the series of figures for these two countries (Table VI) shows that in Indochina the cholera epidemics have a tendency to break out one year later than in Thailand and to last somewhat longer. For instance, the epidemic outbreak of 1925 in Thailand led to an importation of the infection into Indochina, which resulted in an explosive outbreak first in Cambodia in January 1926. While in Thailand this epidemic came to an end during 1929, the incidence of cholera in Indochina continued to be high for another two years.

According to Stowman (1945), Indochina was relatively free from cholera during the war period of 1941-44, but the disease was reported in
May 1945 to be "spreading seriously" in the Mekong Delta and to be descending along the Menam towards Bangkok. As shown in Table VI, the infection continued to be present in Indochina even in 1948, whereas, as noted above, the last epidemic period in Thailand, which had commenced in 1943, terminated one year earlier, in 1947.

Table XI shows that the cholera-affected areas in Indochina are usually those in the south, i.e., Cochinchina and Cambodia, rather than the

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<tr>
<th>Year</th>
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<th>Cambodia</th>
<th>Laos</th>
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<td>cases</td>
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<td>4</td>
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</table>

* From 1947 the figures relate to Viet Nam as a whole.
** From 1947 the figures include suspected cases.
central and northern part of the country comprising Annam, Laos and Tong-king. The 1937-38 epidemic was an exception, since, being connected with an influx of refugees from south China, it was most prevalent in Tong-king and Annam.

Burma

Although Burma is geographically contiguous to the endemic home of cholera in Bengal it is virtually cut off from India by the land route, there being no interconnecting railway communication or any major highway. The sea traffic from the highly infected port of Calcutta to the ports in south Burma is the connecting link, and it is believed to have been responsible for keeping the incidence of cholera high in this country. Indeed, as shown in Table VI, since 1900 not a single year has passed in which cholera deaths did not occur in Burma. The last major epidemics occurred in the years 1915 and 1919. Thereafter, outbreaks of lesser severity were recorded during 1926-29 and in 1932, 1935, 1937, 1940, 1945-46, and 1950-51. Owing to the Japanese occupation of the country, figures for the years 1941-44 are not available.

While Upper Burma is hilly and sparsely populated, the delta of the Irrawaddy River in Lower Burma is low-lying and much more populated. It is, therefore, not surprising to find that, as stated by Norman White (1923), the incidence of the disease is always higher in Lower Burma than in Upper Burma. The delta region of Lower Burma usually suffers most and it is from this area that epidemics appeared to spread.

Although health authorities are often prone to ascribe cholera epidemics to an importation of infection from the neighbouring countries, this has generally not been pleaded in the case of Burma, in spite of the fact that the country lies in close proximity to a principal focus of the disease in Bengal. The Director of Public Health, Burma, for instance, admitted in his report for the year 1937 that cholera has been endemic in the Myaungmya district situated in the delta region of the Irrawaddy. Reference to this district is also made in the following description of the 1934 cholera outbreak, which may be considered as typical for the distribution and spread of the disease in Lower Burma:

"Starting in the Myaungmya district in October, cholera spread to the adjacent districts of Ma-ubin and Pyapon. Bassein became involved in November. These four districts, situated in the delta, are characterized by a network of waterways, with a large proportion of the population living and moving about in boats. The river, in many cases, fulfils the threefold function of a water supply, a washing place, and a latrine. Once cholera broke out, everything favoured its spread and, in a short time, cases were occurring simultaneously in every part of the affected district."

Swaroop & Pollitzer (1952) concluded from a study of the cholera mortality figures for individual districts during the period 1918-38 that a focus of cholera endemicity was situated in Burma within the Irrawaddy
WORLD INCIDENCE

delta in the three low-lying districts of Myaungmya, Pyapon and Ma-ubin. The adjoining districts of Rangoon, Hanthawaddy, Insein, and Bassein also showed a comparatively longer persistence of the disease than the remaining northern districts of Burma. A comparatively minor degree of persistence was noted also in the Thalton districts in the delta of the Salween River.

In Lower Burma, the disease has a tendency to reach its peak during the months of April, May, and June, this period of highest incidence being followed by a decline during the monsoon season and the winter months. The seasonal incidence of cholera in Upper Burma is different, however, the peak incidence being reached during the months of August, September, and October, while a relatively low incidence of the disease is noted during the first six months of each year. The unfortunate result of this seasonal difference is that, once cholera has broken out in Lower Burma, the infection is apt to reach Upper Burma during a season favourable to the epidemic spread of the disease.

Pakistan

For purposes of securing comparability in the series of cholera figures for India given from 1900 onwards, cholera deaths occurring in what is now Pakistan are also shown in the total for India in Table VI. Separate figures of cholera deaths in Pakistan during recent years are shown in Table XII for each province and for the whole country.

TABLE XII. ANNUAL CHOLERA DEATHS BY PROVINCES IN PAKISTAN, 1947-54

<table>
<thead>
<tr>
<th>Year</th>
<th>East Bengal</th>
<th>West Punjab</th>
<th>Baluchistan</th>
<th>North-West Frontier Province</th>
<th>Sind</th>
<th>Total</th>
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</tr>
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</tr>
<tr>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>8,429 *</td>
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</table>

* Preliminary figures.

Consequent to the large-scale movement of refugees between India and Pakistan after the partition in August 1947, cholera broke out in the Punjab and spread to the North-West Frontier Province and Sind. However, apart from this unusual occurrence, cholera in Pakistan has been confined to East Bengal, which happens to lie in the endemic zone of this disease.
The port of Chittagong, situated in this region, has reported cholera deaths annually since 1924, and shows (as indicated in Table XVII) a relatively higher degree of endemicity than any seaport in the East or in Far Eastern countries except Calcutta and Negapatam.

India

The annual cholera mortality-rates per 10,000 inhabitants for what was formerly British India (excluding Burma) from 1877 to 1954 are shown in Fig. 6. For purposes of comparison the cholera deaths reported in Pakistan after the partition year of 1947 have been included in the corresponding rates. However, separate mortality figures for Pakistan have been given in Table XII.

FIG. 6. ANNUAL CHOLERA DEATH-RATE IN INDIA, 1877-1954

In India, cholera has shown a markedly varying incidence from year to year with a maximum of 805,698 deaths in 1900, and a minimum of about 60,000 deaths in the more recent years up to 1953. Earlier, as few as 66,137 deaths had been recorded in 1932. The last major epidemic occurring on the sub-continent reached peak incidence in the year 1943 with 459,930 deaths in what was then British India alone (excluding the States under local rulers). As will be seen later, this unusual rise was attributable in a large measure to severe famine conditions in Bengal, food scarcity in other areas, and other shortcomings caused by the Second World War.

Although in many cases in the past major outbreaks have been confined to a single year, there have been instances when the cholera incidence remained at a high epidemic level for several years in succession. For instance, the epidemic starting in 1875 may be considered to have continued throughout a period of five years until 1879. Similarly, the epidemic of 1927, although not attaining a very high peak, lasted until 1931. The last epidemic which, as noted above, reached its acme in 1943, may be said to have lasted from 1941 until 1945.
At first glance, a scrutiny of Fig. 6 seems to provide indications of some long-term decrease of the cholera incidence in India. In fact, some of the earlier workers, who did not have access to a sufficiently long series of figures, were tempted to conclude that the incidence of the disease was sharply decreasing. Indeed, such a conclusion would be well-nigh inevitable if one were to chart, for example, only the figures from 1900 onwards and, more still, if studies in this direction had been made prior to the 1941-45 epidemic. For then, starting from the highest peak, each successive epidemic up to 1938 would have appeared to be reduced in magnitude. However, the high peak attained by the epidemic in 1943, and its continuance through a 5-year period, leaves room for doubt as to whether conditions have improved enough to bring the cholera epidemics under control. Probably the only conclusion which it seems legitimate to draw is that after the 1919 epidemic the cholera incidence during interepidemic periods has been of a low order in India. It has to be noted in this connexion that some time after the year 1923 public health services were established or expanded in various parts of India, the efforts of which were at first largely devoted to the control of epidemic diseases. It is also important to note that Fig. 6 is based on the total cholera mortality recorded throughout the Indian sub-continent, while the individual provinces show considerable heterogeneity regarding the length and severity of their epidemic outbreaks. Further, Fig. 6 does not provide any indication of a regular periodicity in the incidence of cholera, since—as stated already—a search for periodicity has to be based on figures for areas homogeneous in regard to the epidemiology of the disease.

The peak incidence of cholera in some provinces is, no doubt, partly explained by the holding of large-scale pilgrim festivals, as, for example, the Kumbh fairs taking place every six years at Hardwar, situated in north-west India in Uttar Pradesh (formerly United Provinces), and similar fairs held at different six-yearly intervals at Allahabad in the east of that State. Hardwar and Allahabad are the two most important pilgrim centres, attracting devotees from all over the country. Although Kumbh festivals are held at intervals of six years, alternate festivals, celebrated every twelfth year, have a greater religious significance. However, apart from these occasions, Hardwar is visited by a stream of pilgrims from different parts of India almost throughout the year.

The close association between the Kumbh fairs and cholera incidence is amply shown by the history of cholera in the United Provinces and the adjoining province of the Punjab, which lies farther west. Proof for this contention is furnished by Fig. 7, in which the bars showing the cholera mortality for each year since 1877 have been shaded in different ways so as to indicate the years in which full or half (Ardh) Kumbh fairs have been held at Hardwar or Allahabad. As will be seen, the majority of the years recording increases in cholera mortality were those in which fairs were held in the
province, though in some cases the incidence showed an increase in the year following that in which the fair was held, as for instance in 1880, 1892, 1910, and 1913. There have been, however, striking exceptions, as, for example, in the years 1933 and 1942, during both of which fairs were held and for which only negligible cholera incidence was reported. Such exceptions did occur even earlier, as for instance in 1888, i.e., at a time when cholera control measures during the fairs were not well developed or even altogether non-existent. It is also worth recording that in some years, as for instance in 1887 and 1908, the cholera mortality in the province was unusually high in spite of the fact that no large-scale religious congregations had taken place. While, therefore, Fig. 7 is helpful in illustrating the important role of religious assemblies in giving rise to a high cholera mortality, it also indicates that not necessarily each of these rises must have been the result of situations developing within the United Provinces—the less so because, as noted before, a major cholera-endemic zone is situated to their east.

The association between Kumbh fairs at Hardwar and the incidence of cholera is more clearly evident in the case of the Punjab than in Uttar Pradesh. As will be gathered from Fig. 8, in which the annual cholera incidence in the Punjab from 1901 onwards is shown and where the various bars representing annual deaths are shaded in various ways to indicate the source of the infection, almost all major epidemics in the Punjab were attributable directly to an importation of the disease from Hardwar. An identical opinion was reached by Yacob (1944) who, tracing the origin of cholera outbreaks in the Punjab during the 77-year period of 1867 to 1943,
concluded that almost invariably the manifestations of the disease there were the result of the Kumbh fairs held every six years in Hardwar.

In regard to the second important pilgrim centre in Uttar Pradesh, it was stated by Lal (1937) that

"At Allahabad each and every gathering at the time of the Kumbh and Ardh-Khumb from 1882 to 1918 was accompanied by a great rise of cholera incidence in the eastern districts of the United Provinces, Bihar and Orissa and the Central Provinces."

Lal added:

"The Ratha Yatra fair at Puri (Orissa State), Sinhast fair at Nasik (Bombay State) and Godavari Pushkaram fair at Rajamundry, Krishna Pushkaram at Bezwada, Mahakam fair at Kumbakonam (Madras State) and Sagar mela at the Sundarbans (Bengal) occupy no less enviable positions in relation to cholera epidemics. Some of the epidemics in connexion with these fairs have led to world-wide epidemics."

One might, therefore, be led to believe that in addition to Uttar Pradesh the States of Orissa, Bombay, Madras, and Bengal, where the above-mentioned pilgrim centres are situated, would be particularly liable to suffer from cholera. For the sake of brevity, it is not possible to discuss in detail the part these pilgrim centres have played in propagating epidemics. Sufficient evidence has been quoted, however, to show that, in any study on the periodicity of cholera in India, due attention should be paid to the occurrence of important religious festivals and the holding of large-scale congregations in general.

There can be no doubt that the bulk of the reported cholera deaths in India occurred in what has been called above the major endemic home of the disease, namely in the provinces of Bengal, Bihar, Orissa, Assam, and
the United Provinces. Table XIII, which compares the cholera mortality by decades from 1910 onwards in Bengal, Bihar and Orissa, United Provinces, and Madras Presidency respectively with the corresponding figures for British India as a whole, illustrates this contention. Out of about 10 million cholera deaths which occurred in British India during this period, 29% were contributed by the Bengal Presidency, the population of which was 21% that of British India. Another 25% occurred in Bihar and Orissa, the population of which was 16% of that of British India. The United Provinces and Madras contributed 17% and 14% respectively, the corresponding population percentages being 19% and 16%. Cholera deaths in these four provinces, therefore, were responsible for 85% of the total cholera deaths in British India during the 45-year period of 1910-54.

\[
\begin{array}{|c|c|c|c|c|c|c|c|c|}
\hline
\text{Period} & \text{Bengal} & \text{Bihar and Orissa} & \text{United Provinces} & \text{Madras Presidency} & \text{British India} & \text{Percentage of the total deaths in British India} \\
\hline
1910-19 & 1,004,624 & 563,344 & 678,069 & 615,736 & 3,628,006 & 26 & 26 & 18 & 16 \\
1920-29 & 714,549 & 504,275 & 358,609 & 319,924 & 2,224,755 & 22 & 23 & 17 & 14 \\
1940-49 & 686,535 & 637,319 & 393,270 & 264,248 & 2,110,624 & 26 & 20 & 18 & 13 \\
1950-54 & 126,521 & 83,887 & 36,165 & 82,068 & 380,110 & 33 & 22 & 10 & 22 \\
\hline
\end{array}
\]

* Excluding figures for East Bengal for the year 1946.

When studying the occurrence of major cholera outbreaks in different parts of India, due attention should be paid to the marked differences in the seasons during which the disease is apt to become epidemic in different parts of the country. For instance, even though cholera is believed to be highly endemic in Bengal, its incidence falls to a minimum during the monsoon months of June, July, and August. Then, as stated by Fry (1925) "year after year as soon as the land dries up cholera reappears in mild epidemic form. If there have been tornados and tidal waves bringing salt water into the rivers and tanks, then as in 1876 and 1897 the cholera assumes fulminate epidemic proportions."

The dry season is also of epidemiological importance in so far as it is the season of movement, and there is much emigration and immigration of hired labourers for cutting the winter rice-crop. Nevertheless, in certain non-endemic areas, e.g., in the Punjab, the disease has a tendency to reach
epidemic proportions only during the monsoon months. The seasonal pattern of cholera in India indeed presents interesting features. In Bengal, as already shown in Fig. 2 (page 55), cholera begins to rise in October and November and continues its upward trend to December and January. Thereafter, a decline occurs in February, followed by another rise until the peak is reached in April and May. Although this seasonal pattern holds true for a large part of cholera-endemic Bengal, a more detailed examination, as, for instance, that made by Lal and co-authors (1941), has demonstrated that conditions may differ markedly in different parts of the province. It has already been stated that, as the western borders of Bengal are reached, a curious variation is observed in the months of the south-west monsoons. In Madras Presidency, which is affected by the south-west monsoons in June, July, and August and by the north-eastern monsoons along the Coromandel coast during the winter months of November and December, two peaks occur, synchronizing with the two monsoon periods of the year. Almost all epidemics have adhered to this "time-table" which, in spite of more rapid means of transport in recent years and greater traffic from Bengal north-westwards, has not been altered, possibly because of the consistency of the climatic factors. The presence of two seasons favourable each year to the epidemic spread of cholera probably accounts for the comparatively considerable and persistent incidence of the disease in Madras.

During the period covered by this review, cholera first showed an epidemic increase in 1924 simultaneously in Bengal, Bihar, Orissa, Assam, and the United Provinces as early as March-April, that is, corresponding to the first seasonal peak of the year. In the United Provinces, the first cases of cholera occurred in February, resulting in a violent epidemic at the end of March, which continued through the month of April and was on the decrease from May to July. The onset of monsoon rains in August again increased the incidence both there and in Bihar and Orissa. The wave of infection moved westwards into the Punjab in July, but it did not progress farther north to the North-West Frontier Province. The highest mortality, as usual, was recorded in the middle and upper Ganges valley as well as in Assam. No spread of the infection westwards from India is known to have taken place in 1924, while in the east, only Burma became invaded during that year. With the exception of Korea, the other still cholera-affected countries recorded a lesser incidence of the disease in 1924 than during the previous year.

In the following two years, i.e., 1925 and 1926, the disease was comparatively quiescent in its main centres in India, although in the meantime it is known to have spread in epidemic form to Thailand and Indochina. It has to be noted, however, that the comparative quiescence of cholera in India during 1925 was largely confined to its endemic home in Bengal and to the Indo-Gangetic plains, while at the same time the disease was
responsible for a relatively high mortality both in the extreme north and south of India. Having reached the Punjab in 1924, for instance, cholera spread during the ensuing winter months farther north into Kashmir, where it caused 11,504 deaths during 1925, i.e., practically as many as during the outbreak taking place in that region in 1919, when 11,516 deaths were recorded.

The 1925 outbreak in Kashmir reached its peak in April, and continued until October of the same year, the vast majority of cases occurring in the province of North Kashmir. In the extreme south of India the disease reached its maximum in January 1925 during the north-east monsoon months. The incidence continued at a high level, and another maximum was reached in January 1926. The infection spread farther south to Ceylon.

Epidemic conditions again began to develop early in 1927 in Bengal and Madras Presidency and the cholera incidence was on the increase from April onwards also in the United Provinces and in Bihar and Orissa. The spread westwards rapidly affected the Punjab. While the epidemic showed a tendency to increase in these provinces, in the meantime cholera had spread to the Central Provinces, Hyderabad, and Bombay Presidency, the first two of which were most severely affected in their western districts. With the onset of a favourable season for cholera in Bengal and Madras, the incidence was again at a high level in November 1927, whereas in winter the incidence declined rapidly elsewhere in India. By March 1928, cholera had reached its peak in Bengal and spread rapidly northwards into the Ganges valley through Bihar and the United Provinces to the Punjab, the seasonal rise commencing earlier than usual. In the Madras Presidency also, an increase was observed which, beginning in June 1928, affected mostly the south-eastern districts. By the end of the year, cholera had spread westwards to Travancore State. The worst affected areas, contributing nearly 90% of the total deaths recorded in 1928, were the United Provinces, Bihar, Orissa, Bengal, and the Madras Presidency.

During 1930, cholera remained at an unusually high level in Bihar, Orissa, and United Provinces although it showed a minor decrease in its endemic home in Bengal, while in the Central Provinces and Bombay it prevailed in epidemic form and continued to persist through 1931, declining in 1932 to the lowest level recorded in the previous 60 years. The very low figure of cholera mortality for 1933 is of particular interest in view of the fact that during this year a largely attended Kumbh festival, religiously most important because it was held after an interval of twelve years, took place at Hardwar in the United Provinces. As stated before, as a rule such pilgrimages played a most distressing role in the dissemination of the infection.

The high cholera incidence in India during 1934-35 is attributable to the prevalence of epidemics developing once more in Bengal, Bihar, and Orissa, the United and Central Provinces, as well as in Bombay and Madras.
A sharp increase in the incidence of cholera occurring in 1938 was caused by a wide spread of the infection as the result of an outbreak taking place at the time of the Kumbh fair at Hardwar in April of that year. The consequences of this initial epidemic for the Punjab are shown in Fig. 8 (page 81).

Although the Hardwar festival started on 1 February and lasted till the end of April, the epidemic did not break out until a favourable season had set in. That the progress of the disease from Hardwar northwards was extremely rapid is shown by the fact that, while in the Punjab the first case occurred during the week ending 9 April, in the following week 11 districts up to Lahore had been infected. One week later another 16 districts had become involved and in the following week the North-West Frontier Province had been reached.

The United Provinces, Bihar and Orissa, as well as the Central Provinces also suffered much. The first-mentioned area, bearing the brunt of this epidemic, recorded 70,622 deaths as against only 6341 deaths in 1937. In Bengal, more than twice as many victims were recorded in 1938 as in during the previous year (71,133 deaths as against 32,700 deaths in 1937). However, according to the Public Health Commissioner with the Government of India, this increase was apparently not associated with the Hardwar festival but was due to the occurrence of extensive floods in October 1938, the spread of the disease becoming considerable in the succeeding month of November. The Central Provinces were also severely affected, the cholera mortality there during 1938 reaching a total of 45,332 as against only 1107 deaths in 1937.

The high incidence of cholera during the period 1941 to 1945, in the course of which an unusually high peak was observed in 1943, was probably largely due to wartime conditions which began to exert an influence in India from 1941 onwards. Serious shortages occurred not only in foodstuffs but also in respect of civil medical officers, who had joined the army in large numbers, as well as in regard to medical and sanitary stores, for the procurement of which the army had been given priority. Additional hardship was caused by the invasion of the eastern frontier regions of India by Japanese forces, and still more by the cutting-off of the large rice imports which, in normal times, used to come to India from Burma and other eastern countries. It was under these conditions that a severe famine occurred in Bengal during which, according to conservative estimates, over one and a half million people died. Food scarcity is likewise known to have prevailed in an acute form in several other parts of India, particularly in the south, where extensive and severe cholera outbreaks also occurred.

The increase in cholera incidence during 1941 was first recorded in the Ganges valley, from where the disease spread to the Punjab, Sind, and the North-West Frontier Provinces; the following year showing the same epidemic prevalence.
Out of a total of 459,930 cholera deaths during 1943 in India, Bengal alone contributed 47%, Madras Presidency 25%, Bihar 11%, and Assam 3%. In Bengal, the epidemic was of such a severe nature that it did not even show its normal decline during the monsoon months of July and August, but continued to rise steadily month after month to reach an acme in October.

Even since 1948 cholera has continued to maintain a relatively high level in different States—with the exception of the Punjab where the incidence has shown a consistent decrease—and in some cases even to assume epidemic proportions, as, for instance, in Bengal during 1948-50; Bihar during 1950 and 1952-53; Hyderabad, Bombay and Madhya Pradesh (formerly Central Provinces) during 1952; Madras during 1948 and 1950; and Uttar Pradesh during 1946, 1948 and 1952-53.

The incidence had been high in 1952 in both Bihar and Uttar Pradesh, and in both these provinces it further increased in 1953. A feature of the most recent epidemic—in 1953—was that West Bengal was affected relatively mildly. A westward spread of the infection took place early in 1953 to Madhya Pradesh, Hyderabad and Bombay, where epidemic conditions prevailed throughout the year. The epidemic reached its peak during the months of August and September. A spread occurring further south to Madras was not of the same magnitude as that westwards to Bombay. The disease crossed over to Ceylon in 1953, presumably from the port of Negapatam (now called Nagapattinam) whence sailing vessels had been arriving in Jaffna port, where illegal immigration into Ceylon had been discovered.

It will be clear from the foregoing account that up to the present day Bengal and the adjoining provinces continue to harbour the major endemic focus of cholera in the world. The focus of highest endemicity is, in fact, situated in south Bengal in the delta of the Ganges and the Brahmaputra. A clear demarcation of the endemic zone is shown in Fig. 9. In order to compile this map the annual cholera mortality rates of individual districts in the whole of British India were studied over the period 1901 to 1945. For each district an average death-rate was worked out on the experience of those 15 years which recorded the lowest incidence out of the 45 years. This average cholera mortality rate, called the “endemicity rate”, permits a comparison of individual districts according to their level of endemicity. In Fig. 9, only those districts which showed an endemicity rate of 0.40 per 1000 population or over have been shaded in varying degrees. The four districts in which the endemicity rate exceeds 1.0 per 1000, are Howrah, 24-Parganas, Bakarganj, and Dacca, the last two districts now belonging to East Pakistan. Contiguous with these are the districts of Khulna, Tippera, Faridpur and Calcutta for which the endemicity rates were 0.99, 0.99, 0.97, and 0.92 respectively. Two coastal districts of Orissa State, i.e., Balasore and Cuttack, also show high endemicity rates of 0.91 and 0.81 respectively.
Although all the areas covered with dots in Fig. 1 (page 53) are to be regarded as favourable for the persistence of cholera in varying degrees, Fig. 9 shows that the comparatively more markedly endemic zone is confined mainly to south and south-east Bengal, lower Assam, the coastal districts of Orissa and certain low-lying regions in Bihar along the River Ganges.

A more detailed examination of the cholera mortality figures for subdivisions of individual districts, the thanas, enabled Swaroop (1951) to detect the existence of a major focus within south-west Bengal at the confluence of three rivers, the Hooghly, the Rupnarayan, and the Damodar, in a very densely populated and low-lying tract of land, situated about 40 miles south-west of Calcutta (see Fig. 10). Lying on the opposite bank of the Hooghly River, this focus is almost inaccessible from Calcutta during the
major part of the year on account of floods and poor means of communication. Nevertheless, the proximity of the focus to the city and the persistence of the disease in it at all times are undoubtedly factors responsible for the continued prevalence of cholera in Calcutta from year to year.

In view of the low incidence of the disease recorded during the period 1945-54, it is not unlikely that the zones of cholera endemicity which, as noted before, have been demarcated through a study of the mortality figures prior to 1945, may have shrunk within recent years.

Annual cholera mortality rates for Bengal from 1891 to 1954 are shown in Fig. 11 and the values of these rates for five-year periods are summarized in Table XIV.

In the last column are set out what may be regarded as the index numbers of cholera mortality for each five-year period, the experience of the quinquennium 1891-95 being reckoned as 100. This index number and Fig. 11 serve to indicate a long-term tendency for the decrease of the cholera incidence from the beginning of the century—a trend that was upset during the 1941-43 Bengal famine years. As already stated, the 1943 epidemic occurred under rather unusual conditions, marked by a virtual temporary breakdown of control measures during the famine period. Since

* Based on the average cholera mortality of the five years of lowest incidence.
1944, the incidence has remained at a relatively much lower level as compared with the experience in the previous quinquennia, the frequency of cholera during 1951-55 being only 10% of that in 1891-95.

**TABLE XIV. CHOLERA MORTALITY RATES PER 1000 BY 5-YEAR PERIODS IN BENGAL ; 1891-1955**

<table>
<thead>
<tr>
<th>Period</th>
<th>Mean annual death-rate per 1000</th>
<th>Percentage of the 1891-95 rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1891-95</td>
<td>3.62</td>
<td>100</td>
</tr>
<tr>
<td>1896-1900</td>
<td>2.54</td>
<td>73</td>
</tr>
<tr>
<td>1901-05</td>
<td>3.23</td>
<td>105</td>
</tr>
<tr>
<td>1906-10</td>
<td>2.16</td>
<td>67</td>
</tr>
<tr>
<td>1911-15</td>
<td>2.02</td>
<td>67</td>
</tr>
<tr>
<td>1916-20</td>
<td>1.66</td>
<td>52</td>
</tr>
<tr>
<td>1921-25</td>
<td>1.83</td>
<td>35</td>
</tr>
<tr>
<td>1926-30</td>
<td>1.92</td>
<td>64</td>
</tr>
<tr>
<td>1931-35</td>
<td>1.02</td>
<td>34</td>
</tr>
<tr>
<td>1936-40</td>
<td>0.84</td>
<td>27</td>
</tr>
<tr>
<td>1941-45</td>
<td>1.42</td>
<td>47</td>
</tr>
<tr>
<td>1946-50</td>
<td>0.69</td>
<td>22</td>
</tr>
<tr>
<td>1951-55</td>
<td>0.22*</td>
<td>10*</td>
</tr>
</tbody>
</table>

* Provisional figures.
A study of the rates of decrease in cholera mortality in individual districts of undivided Bengal reveals an interesting pattern. In Table XV, cholera death-rates are shown for each of the 27 districts of Bengal for the two 10-year periods 1901-10 and 1934-43. The last column gives the percentage decrease which has been observed in these two average rates.

The slowest decrease is shown in Chittagong (20%), followed by Faridpur (25%), Tippera (26%), and Bakarganj (28%). All these districts now belong to Pakistan and lie in a highly cholera-endemic area (Fig. 12). The other southern districts of Howrah, 24-Parganas, and Khulna, which also belong to the most endemic area in Bengal, record relatively slower rates of decrease. Table XV suggests that a relatively greater decrease has occurred in the less cholera-endemic zones.

**FIG. 12. PERCENTAGE DECREASE OF CHOLERA MORTALITY FROM 1901-10 TO 1934-43 IN BENGAL**

![Map showing percentage decrease of cholera mortality in Bengal from 1901-10 to 1934-43](image)

### Cholera in Seaports

Annual figures of cholera cases reported in important ports during the period 1926-55 are shown in Table XVI. It would seem that, with the exception of the port towns of India (including Pondicherry in former French India) and East Pakistan, the incidence in seaports has become almost negligible. A study of the cholera-endemic level of various important seaports of south Asia gives the endemcity rates shown in Table XVII.
### TABLE XV. DECREASE IN CHOLERA MORTALITY RATE FROM 1901-10 TO 1934-43 BY DISTRICTS IN BENGAL

<table>
<thead>
<tr>
<th>Districts</th>
<th>Average death-rate per 1000 population</th>
<th>Percentage decrease from 1901-10 to 1934-43</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1901-10</td>
<td>1934-43</td>
</tr>
<tr>
<td>Burdwan</td>
<td>2.65</td>
<td>0.80</td>
</tr>
<tr>
<td>Birbhum</td>
<td>2.89</td>
<td>0.94</td>
</tr>
<tr>
<td>Bankura</td>
<td>1.65</td>
<td>0.77</td>
</tr>
<tr>
<td>Midnapore</td>
<td>3.20</td>
<td>0.83</td>
</tr>
<tr>
<td>Hooghly</td>
<td>2.27</td>
<td>0.61</td>
</tr>
<tr>
<td>Howrah</td>
<td>4.14</td>
<td>1.71</td>
</tr>
<tr>
<td>24-Parganas</td>
<td>4.04</td>
<td>1.70</td>
</tr>
<tr>
<td>Calcutta</td>
<td>2.61</td>
<td>1.09</td>
</tr>
<tr>
<td>Nadia</td>
<td>3.61</td>
<td>1.22</td>
</tr>
<tr>
<td>Murshidabad</td>
<td>2.05</td>
<td>1.09</td>
</tr>
<tr>
<td>Jessore</td>
<td>3.17</td>
<td>1.23</td>
</tr>
<tr>
<td>Khulna</td>
<td>3.09</td>
<td>1.46</td>
</tr>
<tr>
<td>Rajshahi</td>
<td>3.22</td>
<td>0.80</td>
</tr>
<tr>
<td>Dinajpur</td>
<td>0.89</td>
<td>0.25</td>
</tr>
<tr>
<td>Jalpaiguri</td>
<td>0.95</td>
<td>0.49</td>
</tr>
<tr>
<td>Darjeeling</td>
<td>0.52</td>
<td>0.04</td>
</tr>
<tr>
<td>Rangpur</td>
<td>1.40</td>
<td>1.02</td>
</tr>
<tr>
<td>Bogra</td>
<td>2.43</td>
<td>0.66</td>
</tr>
<tr>
<td>Pabna</td>
<td>2.92</td>
<td>1.30</td>
</tr>
<tr>
<td>Malda</td>
<td>2.53</td>
<td>0.60</td>
</tr>
<tr>
<td>Dacca</td>
<td>2.96</td>
<td>1.64</td>
</tr>
<tr>
<td>Mymensingh</td>
<td>3.10</td>
<td>1.30</td>
</tr>
<tr>
<td>Faridpur</td>
<td>2.76</td>
<td>2.09</td>
</tr>
<tr>
<td>Bakarganj</td>
<td>3.47</td>
<td>2.51</td>
</tr>
<tr>
<td>Chattagong</td>
<td>1.54</td>
<td>1.23</td>
</tr>
<tr>
<td>Noakhali</td>
<td>2.63</td>
<td>1.73</td>
</tr>
<tr>
<td>Tippera</td>
<td>2.06</td>
<td>1.53</td>
</tr>
<tr>
<td>Bengal</td>
<td>2.73</td>
<td>1.27</td>
</tr>
</tbody>
</table>

Calcutta heads the list with a rate 4.3 times that of Negapatam and 7.7 times that of the neighbouring port of Chattagong in East Pakistan.

Fig. 13 shows the number of annual cholera deaths in Calcutta from 1841 to 1955. Throughout this period of over a century the city has not
been free from cholera for even a single year, three to four thousand attacks being recorded annually on an average. During the famine year of 1943, Calcutta had 6945 cases but even this high figure was exceeded in two of the more recent years, i.e., 1948 and 1950, during which 7924 and 9529 cases respectively occurred. As shown by Table XVI, the cholera situation continues to be serious at Calcutta and, to a lesser extent, also in Madras.
In order to indicate the months in which the danger of a spread of the infection is potentially greatest, curves of the seasonal cholera incidence in ports where the disease has been more or less frequent in the recent past, are shown in Fig. 14. These curves have been drawn on the assumption that 100 cases of cholera occurred in each of the five ports and show the percentage distribution of cholera cases in 13 four-weekly periods of the year.
TABLE XVII. CHOLERA ENDEMICITY RATES IN VARIOUS SEAPORTS OF SOUTH ASIA, 1946-55 *

<table>
<thead>
<tr>
<th>Port</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcutta</td>
<td>83.8</td>
</tr>
<tr>
<td>Negapatam</td>
<td>16.4</td>
</tr>
<tr>
<td>Chittagong</td>
<td>10.9</td>
</tr>
<tr>
<td>Madras</td>
<td>0.7</td>
</tr>
<tr>
<td>Tuticorin</td>
<td>0.3</td>
</tr>
<tr>
<td>Rangoon</td>
<td>0.2</td>
</tr>
<tr>
<td>Bombay</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* Average annual cholera case-rates per 100,000 inhabitants for the five years of lowest prevalence during the period 1946-55.

The seasonal variation in the incidence of the disease is similar in the ports of Calcutta and Chittagong, showing an increase during the months of May, June, and July. The winter rise is almost negligible. While the port of Chittagong has recorded several weeks free from cholera cases, it is significant to find that in Calcutta city, even though the incidence declines following the monsoons and is low in the winter months, not a single month is known to have been free from cholera during the last half-century.

FIG. 13. ANNUAL CHOLERA DEATHS IN CALCUTTA, 1841-1955 *

* Figures for 1861-64 are not available.
for which monthly figures of cholera cases have been examined. Madras port shows its peak during the months of August and September, and Negapatam during the winter months at the time of the north-eastern monsoons. Bombay port shows a peak during the south-west monsoon period.

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Chapter 3

BACTERIOLOGY

General Remarks

As referred to in the first chapter, several observers had considered cholera to be due to a specific gastro-intestinal infection before proof of this assumption was obtained through the discovery of the *Vibrio cholerae* by Koch in 1883.

Discussing the history of the 1817-19 cholera outbreak in India, Macnabara (1876), himself one of the pioneers in this field, recorded that, according to a conclusion arrived at in 1819 by the Bengal Medical Board,

"the proximate cause of the disease consisted in a pestilential virus, which acted primarily upon the stomach and small intestines; and that the depressed state of the circulatory powers and diminished action of the heart were consequent on the severe shock which the system had received in one of its principal organs."

Basically sound though this concept was, the Board refuted the idea that cholera was a contagious disease and it seems to have been only in 1831 that a *contagium vivum* was incriminated as the cause of the infection by Neale and a few other writers enumerated by Sticker (1912). It would appear, however, that Boehm (1838) was the first who actually claimed to have seen the causative organisms in the dejecta of cholera patients. Since, according to Sticker, Boehm spoke of

"spherical organic particles, which adhered to one another like the parts of a cactus plant and resembled the yeast fungi detected by Theodor Schwann as the causative organisms of wine fermentation" [Trans.],

the claim of this author cannot be accepted. The same is true of similar findings recorded by some workers during the 1849 cholera outbreaks.

Two years after Boehm's findings had been published, Henle (1840), in an essay on miasma and contagion, supported the idea that, with the exception of malaria, the infectious diseases were due to a living contagion, being perhaps caused by minute vegetable organisms (see Greenwood, 1949). An

--- 97 ---
identical view was taken in 1849 by Snow in a pamphlet entitled *On the mode of communication of cholera*. As Snow stated in the second (1855) edition of this publication:

"Diseases which are communicated from person to person are caused by some material which passes from the sick to the healthy, and which has the property of increasing and multiplying in the systems of the persons it attacks."

Applying this concept to the pathogenesis of cholera, Snow came to the conclusion that

"... the morbid matter of cholera having the property of reproducing its own kind, must necessarily have some sort of structure, most likely that of a cell. It is no objection to this view that the structure of the cholera poison cannot be recognised by the microscope, for the matter of smallpox and of chancre can only be recognised by their effects, and not by their physical properties."

A statement even surpassing in importance that of Snow was made in 1849 by Budd who, as summarized by Macnamara, in a letter published on 5 September of that year in *The Times*, expressed the opinion that the causative organisms of cholera were

"a distinct species of fungus which, being swallowed, becomes infinitely multiplied in the intestinal canal, and the action thus excited causes the flux of cholera, which with its consequences constitute the disease."

These organisms, Macnamara continued, Budd believed to be disseminated through society by their contact with food, and principally by the drinking water of infected places; and consequently, he recommended as the most important means of preventing the progress of cholera that the poison which continues to be generated in the bodies of infected persons should be destroyed by mixing the discharges with some chemical compound, such as sulfate of iron or chloride of lime, known to be fatal to beings of the fungus tribe. "As water is the principal means of the dissemination of the disease when it exists, too much care could not be exercised in procuring pure drinking water."

As stated by Sticker (1912), Pacini, examining the intestines of cholera victims at the time of the 1854 outbreak in Florence, claimed to have found a *microbio colerigeno* which had the property of destroying the epithelium and of entering into the deeper layers of the intestine, but not into the blood. Since these bodies, in the warm dejecta, showed a motility by far surpassing the velocity of Brownian movement, they represented, no doubt, a *contagium animale*.

Working at the same time as Pacini in St. Thomas's Hospital, London, Hassall, as quoted by Sticker, found

"myriads of vibriones ... in every drop of every sample of rice-water discharge; of these vibriones many formed threads more or less twisted while others were aggregated into masses which under the microscope presented a dotted appearance."
These vibrios, which were depicted by Hassall and which in Sticker's opinion represented true cholera vibrios, were absent from the blood or urine of the patients, though abounding in their stools.

As claimed by Sticker, in 1866 true cholera vibrios were seen in the dejecta of patients by Leyden (see Wiewiorowski, 1866), and in the vomits as well as in the stools of these sufferers by Bruberger (1867). Similarly, according to a statement made by Virchow at the 1885 cholera conference in Berlin, Klob, in his work (1867) on the morbid anatomy of cholera, depicted and described intestinal organisms obviously identical with V. cholerae.

Feeling convinced of the validity of the views held by Snow and by Budd, Macnamara tried to obtain proof of the presence of cholera germs in the dejecta of the patients by orally infecting experimental animals. As was to be expected, he was unsuccessful and had, moreover, the misfortune of contracting the disease himself, so that he had to go on leave to England preparatory to his retirement in 1876. However, while continuing to work as a surgeon in London, he enlarged his knowledge of bacteriology by studying for some time under Koch in Berlin. Anticipating the 1883 outbreak in Egypt, he applied to the India Office for facilities in order to continue his cholera researches there. It is tragic indeed that, as deplored by Rogers (1950) in a well documented article, officialdom failed to comply with this request, thus bereaving one of the greatest authorities on cholera of the possibility of crowning his lifework by the detection of the germ causing this disease.

However, a French commission, composed of Roux, Straus, Nocard, and Thullier1 as well as a German commission under Koch and Gaffky were sent in 1883 to Egypt. As stated by Chambers (1938) in a fascinating account of their work

"Discovery of the guilty microbe was the goal of each commission, but they approached the problem from different angles. Koch, the pupil of Heue, who was in turn a pupil of Johannes Müller, quite naturally approached the problem as a microscopic anatomist who had turned microbist. He looked for the organisms that were invading the tissues about the intestinal lesions, culturing and isolating them. Roux, the pupil of Pasteur, whose great work in animal diseases had been done by infecting laboratory animals, set out first to reproduce the disease in animals. It just so happened that in this particular disease Roux's method could not succeed because cholera is peculiarly a disease of man and animals do not have it. On the other hand, Koch's method in this particular disease was one of promise."

After the termination of the Egyptian epidemic, continuing his researches in India, Koch found that the peculiar bacilli he had suspected and isolated in Alexandria were invariably present in the dejecta of the cholera patients examined by him in Calcutta, and in the intestines of victims of the disease,

1 Sad to relate, Thullier, a most promising young worker, contracted cholera and succumbed.
FIG. 13. DRAWINGS OF FIRST SLIDE PREPARATIONS BY KOCH OF CHOLERA VIBRIONS *

A. Cross-section of intestinal mucosa of cholera patient. A mucosal gland (a) has been cut obliquely; within it (b) and between the epithelium and the basement membrane (c) are numerous comma bacilli. \(\times 600\).

B. Cover-slip preparation of contents of intestine of cholera patient. The nucleus of the epithelial cell is destroyed (a); a comma bacillus is shown at (b); characteristic grouping of comma bacilli (c). \(\times 600\).

C. Cover-slip preparation of dejecta of cholera patient kept for two days on moist clothing, showing proliferation of comma bacilli, including some S-shaped bacilli (a). \(\times 600\).

D. Cover-slip preparation from edge of drop of pure culture of comma bacilli on meat broth, showing long spiral forms (a). \(\times 600\).

* Reproduced from Koch (1884) by kind permission of the publishers.
but were absent in any other morbid condition. The multiplication of these germs (first called "comma bacilli" on account of their curved aspect when examined under the microscope—see Fig. 15), which regularly took place as the disease progressed, and their disappearance in recovering patients, also lent strong support to the contention that the organisms in question were responsible for the causation of cholera; it still proved impossible, however, to confirm all Koch's postulates by using these organisms to induce the disease in experimental animals and isolating them again from the latter. Nevertheless Koch did not hesitate to report in February 1884 to the German Government that his labours during the cholera outbreaks in Egypt and at Calcutta had been fully successful (see Kleine, 1934).

Though the validity of Koch's findings was soon widely acknowledged, misgivings were expressed because, in contrast to his initial findings made under particularly favourable conditions, it was by no means invariably possible to demonstrate the comma-like bacilli (or, as they were soon called, the cholera vibrios) in individuals who were to all appearances affected with, or had succumbed to, typical cholera. Worse still, findings such as those of Finkler & Prior (1884) during an 1884 cholera nostras outbreak at Bonn soon showed that in addition to the *Vibrio cholerae*, considered unique by Koch, vibrios more or less resembling it do abound and might—as claimed by several observers—be of etiological importance in the causation of gastro-intestinal affections. Indeed, one might claim that from 1884 onwards the study of cholera in the laboratory was to a large extent devoted to endeavouring to differentiate in a sufficiently accurate manner between the true cholera vibrios and cholera-like vibrios. To show to what extent this goal has been reached is one of the main objects of this chapter and some of the later chapters.

**Classification**

According to Bergey's *Manual of determinative bacteriology* (1948) the classification of the cholera vibrio is as follows:

<table>
<thead>
<tr>
<th>Class</th>
<th><em>Schizomyces</em> Nägeli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order</td>
<td><em>Eubacteriales</em> Buchanan</td>
</tr>
<tr>
<td>Suborder</td>
<td><em>Eubacteriinae</em> Breed, Murray and Hitchens</td>
</tr>
<tr>
<td>Family</td>
<td><em>Pseudomonadaceae</em> Winslow et al.</td>
</tr>
<tr>
<td>Tribe</td>
<td><em>Spirilleae</em> Kuyver and Van Niel</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Vibrio</em> Müller</td>
</tr>
<tr>
<td>Species</td>
<td><em>Vibrio comma</em> (Schroeter) Winslow et al.</td>
</tr>
<tr>
<td></td>
<td>(synonyms <em>Vibrio cholerae</em> Neisser; <em>Vibrio cholerae asiaticae</em> Peiffer)</td>
</tr>
</tbody>
</table>

The common characteristics of the genus *Vibrio* (a term derived from the Latin verb *vibrare*, to vibrate) are given in Bergey's manual thus:
"Cells short, curved, single or united into spirals. Motile by means of a single polar flagellum, which is usually relatively short; rarely, two or three flagella in one tuft. They grow well and rapidly on the surface of standard culture media. Aerobic to anaerobic species. Mostly water forms, a few parasites."

**Morphological Characteristics**

**Normal forms**

A description of the appearance of *V. cholerae* under the microscope must be qualified by the following statement: "As long as the usual methods of examination exclusive of flagellar staining are implemented, it is invariably impossible to distinguish between this organism and the allied members of the genus *Vibrio*, because they all appear identical in this respect". In view of the marked pleomorphism displayed by the vibrios in general, and the cholera vibrio in particular, it is at the same time difficult to define the "typical" morphological appearance of the latter. However, as aptly described by Mackie (1929), recently isolated cholera vibrios, which had been grown at 37°C for 18-24 hours on carefully standardized agar media of an adequate alkalinity (e.g., with a pH of 8.0), are apt to appear "as short, definitely curved cylindrical organisms with rounded or slightly tapering ends, and measuring usually 1.5 to 2 μ in length by 0.3 to 0.4 μ in breadth".

It must be realized, however, that even at best the microscopic preparations made from cholera material invariably show some evidence of pleomorphism. Differences in the degree of curvature are bound to be noticeable under all circumstances because naturally those vibrios whose plane of curvature lies parallel to the level of the field of vision, will appear more markedly bent than the organisms lying in other planes (Mackie, 1929). Moreover, individual vibrios, or even strains, may markedly vary in the degree of actual curvature, so that instead of typically curved, more or less straight forms may be present or even predominate. The length of the vibrios also varies from strain to strain so that either longer (and invariably slightly curved), or short (and markedly bent), forms are conspicuous or solely present. Even in recently isolated cultures occasionally quite short forms resembling coco-bacilli are found (see, for example, Seal, 1935) (Fig. 16, 17 and 18).

Shorter or longer forms resembling in appearance the letter "S" are often seen. The occurrence of the former is due to the fact that the vibrios are not merely bent in one plane, but also twisted, thus representing a part of a screw turn (Kolle & Prigge, 1928), while the longer "S" forms are the result of the adherence of two vibrios, specially those which have not parted after transversal fission. Such adherent vibrios may, however, not only appear in the "S" form, but may also form semicircles. In old cultures, long spirals, due to the adherence of several vibrios, may be conspicuous, but
FIG. 17. NORMAL FORM OF VIBRIO CHOLERAE, SHOWING CONDENSATION OF PROTOPLASM AND IMPLANTATION OF FLAGELLUM WITH COCCAL FORM AT RIGHT OF PHOTOGRAPH

Reproduced by kind permission of J. Giuntini, Institut Pasteur, Paris.

Electron microscope photograph: x 50,000.

FIG. 18. GIANT COCCAL FORM OF VIBRIO CHOLERAE WITH FREE FLAGELLA

Reproduced by kind permission of J. Giuntini, Institut Pasteur, Paris.
FIG. 16. 18-HOUR CULTURE OF VIBRIO CHOLERAE ON NUTRIENT AGAR

Reproduced by kind permission of J. Gallut, Institut Pasteur, Paris.

Cilia stained by Van Ermengem method. x 1000.
such spiral filaments are absent in films made from recently isolated cultures. The occasional presence of such spirals in smears made from the dejecta of patients is apt to be due to contamination rather than to the adherence of true vibrios (Gruber, 1887).

It is noteworthy that in smears made from the flakes of typical, rice-water-like cholera stools the vibrios often show a characteristic arrangement, lying with their long axes parallel “like fish in a stream”, this being probably due to the viscosity of the mucus in which they had been embedded (see Sticker, 1912).

While the vibrios seen in stool smears, though apt to vary in their dimensions, usually show the typical curvature, those in histological sections of the intestines as a rule have the appearance of short straight rods. For this reason and also because spindle-shaped forms may be present, the vibrios in such sections may resemble glanders bacilli (Malleomyces mallei), a feature noted by Koch in his initial investigations.

As far as the findings in smears from cultures are concerned, it is important to note that the above-described “typical” appearances of some of the organisms seem indicative merely of a phase in their development. Henrici (1925), who studied this problem with particular care, distinguished between (a) an embryonic stage corresponding to the period of accelerated growth, in which the vibrios were large and bacillary in form; (b) an adult stage, characteristic of the period of a decreasing growth-rate, during which typical vibrios were found; and (c) a senescent stage during which the irregular forms described below became apparent or even predominant.

Earlier observations made in this connexion by Wherry (1905) showed that such morphological differences indicating successive growth phases may be demonstrable simultaneously in one and the same culture: at the periphery of the growth, where active cell division took place, the vibrios displayed a short and almost oval form, whereas the older forms in the centre of the growth tended to be more elongated and to undergo involution.

Envelopes and capsules

Observations suggestive of the formation of an envelope or capsule by the V. cholerae seem to have been made under exceptional circumstances only.¹ Using the flagellar stain recommended by Yokota (1924, 1925) to study the opaque variant of this organism, Balteanu (1926) found that some of the vibrios stained in this manner were surrounded by a thick layer of pink-staining material. When, instead of using this stain, the films were coloured for two minutes with carbol fuchsin, then treated for 10 to 20 seconds with 1% hydrochloric acid and washed with water,

¹ See, however, the statement of Kofe & Frigge (1928), quoted later in the section dealing with the staining properties of V. cholerae (page 105).
the presence of a thick envelope stained pink surrounding the red vibrio was easily demonstrated. Sometimes a like covering enclosed two or several bacteria in a common matrix of mucus-like material. The opaque variant had evidently acquired the faculty of producing a slimy exudate simulating a capsule.

Further observations on this point have been recorded by Bruce White (1938) in a most valuable study on the rugose variant of V. cholerae. He stated that he had seen truly capsulated forms only in the case of a markedly atypical strain, whereas, generally, the organisms composing the rugose colonies appeared to be enclosed in a common zoogloe of gelatinous or mucoid intercellular material. However, ascribing the rugose condition of the cholera vibrios to an intensification of normal secretory processes, Bruce White maintained that no fundamental, but merely a gradual, difference existed between the truly capsulated forms and those embedded in a common matrix.

Flagella

Most cholera workers are in full agreement with Koch's original statement, confirmed by early observations in Egypt (see Kolle & Gotschlich, 1903) and ample subsequent work in India, that, in contrast with part of the cholera-like species, V. cholerae possesses only one polar flagellum. Statements made to the contrary by a few observers deserve no credence, because they were never based upon results obtained with strains freshly isolated from patients but upon findings made in the case of growths which had undergone the vicissitudes of prolonged storage, in variants, or in vibrios from carriers (see Seal, 1935). More important still, most, if not all, claims that the cholera vibrio may possess more than one flagellum were made before the now-available reliable methods of serological identification could be implemented.

As summarized by Mackie (1929):

"the length of the flagellum is somewhat variable, measuring up to 4 or 5 times the length of the vibrio. Long flagella are frequent but short vibrios with short flagella may be seen. Kolle & Prigge (1927) have figured these two morphological types, namely short ovoid organisms with short flagella and longer forms with long flagella."

Motility

It is generally agreed that, if examined under the conditions specified below, the cholera vibrios present in the dejecta of patients or in recently isolated cultures are invariably motile, showing a "scintillating" movement, compared by Koch to the exceedingly rapid progress of a host of gnats, and sometimes also exhibiting a "centrifuge" movement, consisting of a rotation on their short axes. Studying the rapidity of its locomotion, Sanarelli (1919) found that V. cholerae was endowed with a speed three times greater than that of Bacillus prodigiosus, five times that of Salmonella typhosa, ten times that of Escherichia coli, and twelve times that of B. megatherium.
In conformity with a general rule, the cholera vibrio is most actively motile at a temperature of 37°C. Movements are slower at lower temperatures and cease altogether at 16°C. Riemsdijk (1929) found that the motility of cholera vibrios taken from the fluid beneath the surface pellicle of broth cultures was apt to be rather slight as compared to the active movements of the organisms collected from the pellicle. It appeared, therefore, that differences in oxygen supply exerted an important influence on the speed with which \textit{V. cholerae} progressed.

As noted by some workers, the organisms composing cultures which had undergone prolonged storage, or variation leading to the formation of atypical colonies, may show a loss of motility. Nobechi (1923), who found two such immotile strains among the 88 stock cultures of \textit{V. cholerae} he examined, established that this loss of motility was due to the absence of flagella. Balteanu (1926), who like Baerthlein (1912) before him described a variant of the cholera vibrio characterized by the formation of opaque colonies and as a rule also by complete loss of motility, was of the opinion that the production of a slimy exudate by the vibrios in question explained "to some extent" the latter phenomenon. It is important to note, however, that the organisms were found to possess no flagella.

\textit{Modes of multiplication}

Elongation of the vibrios, followed by transversal fission, is undoubtedly the usual, and according to most workers, even the only mode of multiplication of \textit{V. cholerae}. However, Braulke (1933) felt certain that longitudinal division of the organisms may also occur. The claim that reproduction may be affected as well by the formation of gonidia will be assessed in a later part of this chapter.

\textit{Staining properties}

It is certain that \textit{V. cholerae}, which stains readily with all usual laboratory dyes, is, as Mackie puts it, "definitely and uniformly Gram-negative". The validity of this statement was corroborated by the investigations of Braulke, who was able to establish that the occurrence of Gram-positive cocci in vibrio cultures, instead of indicating the presence of the C forms postulated by Kuhn & Sternberg (1931), was actually the result of latent contaminations.

As will be discussed in a later chapter, several of the flagella-staining methods have been found useful in cholera laboratory work. An interesting statement made in this connexion by Kolle & Prigge (1928) was that the cholera vibrios stained according to these methods appear much thicker than those stained in the usual manner, because owing to the use of mordants for the former purpose, the teguments as well as the bodies of the organisms become stained.
Nuclei, granules, and filtrable stage

Discussing the morphological characteristics of the cholera and allied vibrios, Peruzzi (1926) stated that these organisms may show differentiated chromatic bodies, presenting during division not only the appearances, but the behaviour of a nuclear apparatus. Korobkova (1931, 1936), studying the morphological aspect of cholera vibrios grown on a special potato-starch medium, also noted the occurrence of well-differentiated nuclei.

In a recent publication, Paoletti (1952) claimed to have demonstrated, with the aid of the method of Robinow (1942, 1944), chromatic bodies in cholera vibrios similar to those described in other bacteria and considered by some workers to represent morphologically discrete nuclei.

The presence of polar bodies in cholera vibrios has been noted by several observers, first apparently by Finkler & Prior (quoted by Sticker, 1912) who reached the opinion that these Polkörper, becoming liberated and sedimented after decay of the vibrios, could give rise to typical growths even after they had been subjected to prolonged exsiccation. Similarly, Hüppe (1885) postulated that the granules observable in cholera vibrios which had become transformed into filaments after exhaustion of the media, could assume the role of resistant "arthrospores" and thus become a link in the propagation of these vibrios. However, as stressed by Kolle & Prigge (1928), subsequent observations showed that cultures rich in such granules were no more resistant to exsiccation or other untoward influences (heat, disinfectants) than growths free from "arthrospores".

A similar view was also expressed by Braulke (1933) who found that the small spherical forms of the vibrios (Kügelchen), apt to become preponderant in old cultures, specially those constantly kept at 37°C, and suspected by some workers of acting as "gonidia", were actually unable to multiply.

Braulke, like Bisceglie (1929) before him, was also unable to find evidence for the existence of a filtrable stage of the cholera vibrio, which had been postulated by a few workers. In the opinion of Braulke, defects in the filter-candles they had used were responsible for the apparently positive results reported by these observers.

Morphological variation

Ample experience has shown that the cholera vibrios in old cultures as well as those grown in the presence of substances apt to prove inimical to their development, often display a morphological appearance markedly different from that found upon examination of fresh material. As stated by Mackie (1929), under these circumstances

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1 The morphological changes resulting from dissociation of the *V. cholerae* will be described in a later section of this chapter.
A variety of shapes may be observed, e.g. straight organisms, thicker and swollen individuals, spherical forms with faintly stained centres, spindle-, club-, and pear-shaped organisms, individuals with irregular swellings, long spirals measuring up to 17μ, and cells which present a completely distorted structure.

In addition to these forms, Mackie referred also to the observation of spherical or triangular giant forms, branched filaments, cladothrix-like forms, and "budding" forms with roundish protuberances.

In a recent article, describing the morphological changes taking place when cholera vibrios were kept in penicillin solutions (25-100 units/ml), Bruce White (1950) noted the appearance (a) of numerous globular forms at first 8-10μ in diameter and motile, but enlarging upon prolonged incubation, losing their motility as well as their staining properties, becoming vacuolated and apt to bulge in subsidiary masses from the periphery; (b) under optimal conditions, also of star-fish-like forms with tapering branches. As noted by Bruce White,

"on staining the broader part of the branches is seen to consist of double or multiple chains of nucleus elements; the finer branches are formed by single vibrios and from these the culture may regenerate, either in its original form, or, if seeded on to fresh penicillin-agar, in the spherical forms".

The question of what generally causes the above described morphological changes has been the subject of considerable debate. The contention of a few authors that some of the abnormal forms, particularly the "budding" forms, might play a role in the perpetuation of V. cholerae has been generally refuted. In fact in the opinion of many observers these atypical forms were invariably the result of involution. Mackie, who was among those advocating the latter view, supported it by pointing out that these forms

"occur in cultures of some duration after growth has stopped and many of the individual organisms are dying and autolysing", and adding that "the various irregular forms described are such as might reasonably be expected to result from cell degeneration and particularly autolysis following death".

There can be no doubt that the above-described changes in morphological appearance are often the result of involution, the less so as it was sometimes possible to establish that the irregular forms which had developed under unsuitable conditions were incapable of multiplication. At the same time, however, it would not be justifiable to claim that processes of involution play an exclusive role in this respect, since evidence is available to show that cholera vibrios which had become morphologically atypical because they had been subsisting on exhausted media or in the presence of substances inimical to them, were capable of reverting to type when grown once more under suitable conditions. Particularly illuminating observations in this respect have been recorded by Braulke (1933) and, recently, by Paoletti (1952).
The former worker, replanting cholera vibrios previously grown on media containing lithium chloride on plain agar, noted that at first mainly the abnormal "lithium forms" (small or large spheres, etc.) developed on the latter medium but that upon continued subcultivation these aberrant forms became rarer and were gradually replaced by typical vibrios.

More convincingly still, Paoletti, implanting material from old cholera cultures on new media and examining these subcultures at two-hourly intervals, was actually able to observe that the originally present round forms assumed first a quadrangular and then a sausage shape, the latter forms becoming afterwards elongated, and finally converted into typical vibrios.

These and similar observations leave no room for doubt that the appearance of morphologically atypical forms of *V. cholerae*, besides being the result of involution or, as will be shown later, of dissociative processes, may be also indicative of a temporary adaptation of the organisms to unsuitable conditions (*Anpassungsformen* of the German writers).

Cultural Characteristics

Growth limits and requirements

*Reaction of the media*

When dealing with the reaction of the media suitable for the cultivation of *V. cholerae*, attention must be devoted to (a) the initial pH of the fluids or solids used for this purpose, and (b) the changes in the reaction of the media taking place in the course of cultivation. As will be shown later, a high initial pH is of great importance for the primary isolation of the organisms, while in addition to this, a maintenance of not too low a hydrogen-ion concentration exerts a great influence, when cultivation of the vibrios in bulk is called for, as for instance in the course of vaccine preparation.

*Initial pH.* Though, as summarized by Pollitzer (1934b), "the *V. cholerae* is not among the micro-organisms demanding elaborate preparations for their cultivation", it proves indiscriminating in this respect only as long as the media used for its growth possess a suitable pH. The presence of even a slight degree of acidity, corresponding, according to Kitasato (quoted by Kolle & Prigge, 1928), to 0.07% HCl, is sufficient to impede the multiplication of the cholera vibrios. A markedly high alkalinity of the media, on the contrary, is not only well tolerated by these and many other vibrios, but even proves most beneficial because it counteracts the growth of the contaminating bacteria often present in the specimens coming for examination.

It is not surprising to find that some variance exists in the statements made by different observers regarding the pH limits within which satis-
factory growth of *V. cholerae* is apt to take place, because they worked with different, or at least with differently prepared, nutrient media. Of great importance in this respect is also that, as shown by Kabelik & Freudmann (1923), the suitability of the media for the growth of the cholera vibrio depends upon an interrelation between the hydrogen-ion concentration and the NaCl content, the optimum of the latter becoming lower as the former increases, and *vice versa*. As far as the plain media routinely used for cholera diagnosis, particularly peptone water, are concerned, however, modern workers recommend an initial pH ranging from 8.0 to 9.0 or slightly higher (9.5 according to Matsuo, 1924). Vedder & Van Dam (1932), assessing the value of Dieudonné agar (a selective medium described in a later chapter) for the cultivation of *V. cholerae*, found that no growth took place if the pH of the plates exceeded 9.6. This is fairly well in accord with the experience of Read et al. (1939) when growing cholera vibrios in 1/100 peptone water with 1% NaCl, according to which multiplication occurred up to a pH just in excess of 9.4, but with some reduction from 9.4 upwards. In the opinion of these workers

"... a pH of 9.2 may, therefore, be taken as the limit for satisfactory multiplication, a figure which is supported by the results of experience in isolating the vibrio from natural sources."

**pH changes during cultivation.** That cultivation in the usual glucose-containing media leads to a marked lowering of the pH and that such a drop even takes place in the case of media into which no fermentable sugars have been incorporated is shown by the observations of Banerjee (1939) recorded below:

<table>
<thead>
<tr>
<th>Hours (or days) of growth</th>
<th>Ordinary broth</th>
<th>0.2% glucose broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8.2</td>
<td>7.6</td>
</tr>
<tr>
<td>4</td>
<td>8.0</td>
<td>7.4</td>
</tr>
<tr>
<td>6</td>
<td>7.6</td>
<td>7.2</td>
</tr>
<tr>
<td>8</td>
<td>7.6</td>
<td>7.0</td>
</tr>
<tr>
<td>10</td>
<td>7.6</td>
<td>7.0</td>
</tr>
<tr>
<td>12</td>
<td>7.6</td>
<td>7.0</td>
</tr>
<tr>
<td>24</td>
<td>7.6</td>
<td>7.0</td>
</tr>
<tr>
<td>30 days</td>
<td>7.4</td>
<td>6.8</td>
</tr>
</tbody>
</table>

*Remarks.* (a) The drop of the pH was uniform regardless of whether aerobic or anaerobic cultivation was resorted to. (b) Banerjee found that, in contrast to the changes recorded above, no lowering of the pH took place when cultivation in Ramon's glucose medium (prepared from an HCl digest of hog's stomach and minced veal) was resorted to, the pH remaining at 8.2, apparently the original level.

Read et al. (1939) found that a pH of 6.0 marked the lower limit of the range within which multiplication of the cholera vibrio took place in peptone water. Similarly, Jennings & Linton (1944a), working with a medium which contained a casein digest besides inorganic salts and varying amounts of glucose, found that most rapid growth of *V. cholerae* took
CHOLERA

place while the pH was falling from about 8.5 to 6.0, while lower as well as higher values were associated with inferior growth rates. Considering their experiences, Jennings & Linton suggested

"that while a high initial pH may be optimal in the sense that it gives the best conditions for an extended period of growth, the most desirable pH for rapid multiplication may lie in the region near neutrality. Experiments showed that a pH of 10 was injurious to the vibrio and usually prevented growth altogether. Invariably growth stopped when a pH of 5.5 was attained, whether at the end of a long vigorous growth starting at high pH or at the end of a shorter period when growth was initiated at a lower pH level."

Various procedures have been used to counteract the lowering of the pH which takes place in the course of the cultivations of *V. cholerae*. Some workers resorted to the periodic addition of alkalis to the cultures, Hirsch (1928), for instance, using calcium carbonate for this purpose, Veeraraghavan (1949) sodium bicarbonate, with the aid of which it was possible to maintain the pH of the special medium he employed (see later) at a pH level of 8.0 with a marked growth increase. Various buffer substances have been incorporated in the media by other workers. Jennings & Linton (1944b) who, as will be described below, worked with a simple medium containing a casein digest besides glucose, used "aeration" with a mixture of air and CO₂ to maintain the pH of their cholera cultures at a satisfactory level. The efficacy of this procedure was confirmed by Ranta & McLeod (1950).

Gallut (1947a) admitted that vigorous bubbling of air through the media promoted the growth of *V. cholerae* by maintaining the oxidation-reduction potential of the organisms at a constant level even in the presence of glucose, which in this case was oxidized instead of being fermented. However, in a subsequent paper (1947b), he adduced doubts whether cultivation under enforced aeration was advantageous for the purposes of cholera vaccine manufacture because the vibrios grown with the aid of this procedure showed an atypical morphology (prevalence of filamentous forms and early appearance of global forms) as well as diminution of their nitrogen content.

Recently studying again the growth and survival of the cholera vibrio in relation to pH, Sarkar & Tribadi (1954) drew attention to the fact that

"Acid production by the vibrio which determines the pH of the culture fluid depends (apart from other factors such as presence of fermentable sugar or other constituents, amount of growth, etc.) on the available surface area of the medium and therefore oxygenation. pH of 24 hours' broth culture of *V. cholerae* in 30 cc. nutrient broth of pH 7.6 in a test tube (1½ inches diameter) became 6.1, whereas pH of the same in Roux flasks (5½ inches diameter) became 8.2. Growth was also much heavier in the latter."

Further reference to the pH adjustment of the media used for the cultivation of *V. cholerae* will be made in chapter 7, where the practical aspects of cholera laboratory work will be discussed.
Salt requirements

Beauverie (1916), cultivating cholera vibrios in broth with NaCl concentrations of 7, 9, 15, 20, 30, 50, 90, and 100 per 1000 respectively, found growth to become visible within 24 hours in all concentrations up to, but not above, 50 per 1000. To judge from the formation of a particularly thick pellicle, an NaCl concentration of 30 per 1000 was particularly favourable for the multiplication of the organisms. However, Beauverie found that the cholera strains cultivated in broth with an NaCl content of 30-50 per 1000, while being at first favoured in their growth, aged quickly, showing within a few days evidence of involution and loss of motility.

As noted by Kabelik & Freudmann (1923), Sierakowski, in an article published in the Przeglad epidemiologii in 1922, had recorded that an NaCl concentration of 0.5% was optimal for the growth of V. cholerae. In Sierakowski’s opinion, the discrepancy between his findings and those of Beauverie was due to the fact that, in contrast to Beauverie, he had worked with solid media. However, Kabelik & Freudmann, making comparative tests with 2% peptone water, ordinary broth, agar, and gelatin with a varying NaCl content (0-6%), found that no fundamental differences existed between the solid and fluid media, as assumed by Sierakowski. Like Beauverie they recommended the use of peptone water with an NaCl concentration of 3% for practical cholera laboratory work—a proposal also made more recently by Genevray & Bruncau (1938d).

Valuable investigations on the salt requirements of Vibrio cholerae were carried out by Read et al. (1939), who mainly used an artificial concentrated sea-water solution for their tests, but also experimented with the components of this preparation (sodium chloride, magnesium chloride, magnesium sulfate, and potassium chloride) and with some other substances.

Read and his colleagues established in the course of these investigations that

“in the absence of salt multiplication did not occur in any peptone concentration and in no case did survival reach 24 hours”.

Multiplication of V. cholerae was observed in the case of 1/50 000 peptone water at sea-salt concentrations of 0.5% to 3%, in the case of 1/5000 peptone water at a salt concentration as low as 0.1%, in that of 1/500 peptone water even at a salt concentration of 0.075%.

Testing individual salts (calcium chloride, sodium nitrate, and sodium sulfate, as well as the above mentioned) Read et al. found that “any one of the salts tested except magnesium sulphate can promote multiplication, but none seems to do so in any specially low concentration,” and that the sea-water solution mainly used for the experiments had a somewhat
higher capacity for promoting multiplication than the individual salts tested.

Magnesium sulfate, besides being shown to be incapable of promoting multiplication or survival of \textit{V. cholerae}, was also found to be rapidly lethal to the vibrios in the higher concentrations tested. Sodium sulfide (\(\text{Na}_2\text{S}\)), on the other hand, if added to \(1/50\,000\) peptone water in a strength of \(0.0003\%\), secured multiplication of the cholera vibrio at a sea-salt concentration of \(0.05\%\), while \(0.00003\%\) \(\text{Na}_2\text{S}\) was sufficient to secure growth of the vibrios at a sea-salt concentration of \(0.1\%\).

\textit{Oxygen requirements}

The dependency of the cholera vibrio on the presence of oxygen attracted the attention of Koch (1884), who placed pieces of mica on gelatin plates inoculated with \textit{V. cholerae}, and noted that the practical absence of growth under these platelets stood in marked contrast with the abundant development of the organisms round them. The early observers were also impressed by the fact that, when cultivated in fluid media, the vibrios grew most abundantly at the surface of these, and were led to believe that an avidity of the organisms for oxygen fully explained why, typically, a pellicle formed on the surface of these cultures. Hesse (1893), carrying out gas-analytic studies, reached the conclusion that the \textit{V. cholerae} was unable to grow in the total absence of oxygen.

However, carrying out studies on the oxygen requirements of the cholera vibrio, Hirsch (1926\textit{a}) found that this organism was able to grow anaerobically as well as aerobically in a simple chemically-defined medium, provided that a fermentable sugar (glucose) had been added to the latter. These observations, he maintained, went a long way to explain how the vibrios could multiply in the intestine under what amounted practically to anaerobic conditions.

Carrying out further investigations on the metabolism of the cholera vibrio under aerobic and anaerobic conditions, Hirsch (1928) reached the following conclusions:

1. The aerophilic behaviour of the \textit{V. cholerae} in carbohydrate-free amino-acid solutions or in peptone solutions is conditioned in an obligatory manner by the chemical composition of the substrate and cannot be considered a specific property of the organism.

2. The \textit{V. cholerae} is capable of multiplication under strictly anaerobic conditions provided that carbohydrates are available as an anoxybiotic source of energy.” [Trans.]

Working with broth media, Banerjee (1939) fully confirmed the contention of Hirsch that the \textit{V. cholerae} was capable of growing luxuriantly under anaerobic as well as under aerobic conditions in the presence of glucose. Moreover, comparing the growth of this organism in ordinary broth tubes and in tubes in which a layer of vaseline oil had been super-
imposed, Banerjee found that a restricted growth of *V. cholerae* took place in glucose-free broth even when the access of air to the culture medium had been prevented. The evidence Banerjee obtained in this respect may thus be summarized:

<table>
<thead>
<tr>
<th>Hours of growth</th>
<th>Growth of <em>V. cholerae</em> in millions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aerobic</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
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<tr>
<td>6</td>
<td>500</td>
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<td>9</td>
<td>2200</td>
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<tr>
<td>12</td>
<td>4500</td>
</tr>
</tbody>
</table>

In view of the evidence adduced above, the cholera vibrio must be considered a facultatively anaerobic, rather than an obligatorily aerobic, organism.

**Temperature requirements**

In contrast to certain other vibrio species (e.g., the one found to be the causative organism of a fish epizootic by David (1927), which grew best at 8°-20°C), multiplication of *V. cholerae* is most abundant within a temperature range of 30°-40°C with an optimum at about 37°C. Growth at 22°-25°C, i.e., at temperatures used mainly for the incubation of gelatin plates, is still fairly satisfactory.

As claimed by Koch (1884), the cholera vibrios are unable to multiply at temperatures below 16°C. However, as summarized by Kasansky (1895), it was soon shown by some other workers that a slow growth of *V. cholerae* may still take place at temperatures ranging from 8°C to 15°C. In Kasansky’s own experience a multiplication of the organisms still occurred at 10°C to 12°C. Moreover, as will be seen in the concluding part of this chapter, the cholera vibrios show a most remarkable resistance to low temperatures, which are apt to prolong the life-span of the organisms even though no longer permitting their multiplication.

**Nutritional requirements**

The efforts made by a number of workers to determine basic nutritional requirements of the *V. cholerae* by cultivating it in simple, chemically defined media, may be said to fall into two categories: (a) attempts to grow this organism in media containing only ammonium salts but no amino-acids; (b) use of media in which amino-acids were the essential constituents. These two classes of investigations will now be dealt with seriatim.

(a) *Ammonium salts*. Anderson, in *An introduction to bacteriological chemistry* (1946), stated that, like certain other bacteria, the vibrios comprise two types of strains: (1) "exacting" strains, which require amino-acids for their growth, and (2) "non-exacting" strains, capable of growing on ammonium salts as well as on amino-acids. Anderson added that "the 'exacting' strains are usually pathogenic".
The evidence available in this respect regarding the V. cholerae may thus be summarized: Kisch (1919), in contrast with some other early observers, was able to cultivate this organism on a basic agar medium to which 0.19% ammonium sulfate or 0.262% ammonium tartrate had been added, while he obtained no growth on the basic agar alone. He postulated, therefore, that the cholera vibrio was facultatively capable of growing in the presence of ammonium salts instead of organic nitrogen compounds.

Linton & Jennings (1944) and Jennings & Linton (1944b), who cultivated cholera vibrios in media containing, besides ammonium sulfate, other organic salts and glucose, as well as either peptone or a casein digest, came, on the contrary, to the conclusion that ammonium sulfate acted as a buffer rather than as an essential nutrient. As stated by Linton & Jennings, growth took place if this chemical had been omitted from the media, while, on the other hand, V. cholerae failed to grow in the presence of ammonium sulfate but the absence of either peptone or casein digest solution. However, in a later paper, Jennings & Linton (1944b) admitted that they had found “that very good growth could be obtained occasionally when no nitrogenous matter other than supplied by the inoculum was incorporated in the medium. The irregularity of results, however, prompted us to include the additional casein digest as a routine procedure, since the material could be completely removed by dialysis when desired.”

It is of great interest to note that recently Saxena et al. (1953) recorded constant success when cultivating 14 cholera strains as well as one El Tor strain, one strain of water vibrios, and one “rough” (? cholera) strain in a medium of pH 8.0 made up according to the following formula:

- Ammonium phosphate (NH$_4$H$_2$PO$_4$) 0.1 g
- Glucose 0.1 g
- Sodium chloride (NaCl) 0.5 g
- Magnesium sulfate (MgSO$_4$·7H$_2$O) 0.02 g
- Dipotassium hydrogen phosphate (K$_2$HPO$_4$) 0.1 g
- Distilled water to make 100 ml

*Note.* Ammonium phosphate was found to give a better yield after 24-hour incubation at 37°C than ammonium sulfate.

While the observations of Saxena et al. prove that cholera vibrios can utilize ammonium salts as their sole nitrogen source, it is important to note that growth in the above-mentioned medium took place only when glucose was present and when large inocula (“at or above 100 million of organisms per cc. of medium”) were used.

The observations of Saxena and colleagues were confirmed by Bhaskaran & Rowley (1956) in that these two workers found that out of a total of 158 vibrio strains (including 148 cholera strains) half were able to grow on a simple inorganic medium, which, besides containing ammonium ions as
the sole source of nitrogen, also contained glucose. The other 79 strains (including 71 cholera strains) required in addition purines for their growth; hypoxanthine being the simplest of these substances which proved adequate. It was further established that the purine-requiring strains were capable of growing on inorganic media if human, rabbit or goat serum was incorporated. The sera of rats, mice, guinea-pigs or horses failed to support the growth of these strains.

(b) Amino-acids. As summarized by Hirsch (1926b), Uschinsky (1893) was the first to cultivate cholera vibrios in a chemically defined fluid medium which contained NaCl, calcium chloride, magnesium sulfate, and dipotassium hydrogen phosphate, as well as glycerol, ammonium lactate, and sodium aspartate. Fraenkel (1894) established, however, that out of these substances only three, namely, NaCl, dipotassium hydrogen phosphate, and a salt of aspartic acid were indispensable for the growth of V. cholerae. Hirsch (1926b), making further careful studies of this subject, found that this organism was able to use l-aspartic acid as the sole nitrogen and carbon source for its metabolic and energy requirements, and that the decomposition of this acid was the result of an oxidative process, the end products of which were ammonia, acetic acid, and carbonic acid.

Further exhaustive investigations in this field were recently undertaken by Ranta & McLeod (1950), who tested 20 different amino-acids by adding them singly, or in combination, to a basic medium containing 5 g of sodium chloride, 0.75 g of dipotassium hydrogen phosphate, and 0.1 g of magnesium sulfate in a litre of distilled water. While confirming that asparagine gave relatively the best results, if used singly, combinations of two or several amino-acids proved more satisfactory. Ranta & McLeod recommended ultimately a medium containing 0.067% tyrosine, 0.051% glycine, 0.042% asparagine, and 1% glucose.

Agarwala, Krishna Murti & Shrivastava (1953) established in the course of a recent study on the oxidative metabolism of cholera and allied vibrios that cysteine, threonine, and asparagine were oxidized as at fast a rate as glucose, lactate, and pyruvate. Basic amino-acids showed very little oxygen consumption.

Accessory growth factors

As will be gathered from the statements made above, accessory growth factors ("bacterial vitamins") are not indispensable for the cultivation of the V. cholerae. However, Veeraraghavan (1949), using a chemically defined medium which contained, besides different salts, ammonium sulfate and l-cystine, noted that addition of Marmite (an autolyzed yeast product containing anti-neuritic vitamin) had a growth-promoting effect. Substitution of this substance by thiamine hydrochloride, nicotinic acid, pyridoxine hydrochloride, calcium panthothenate, riboflavin, biotin, and
yeast nucleic acid, singly or in combination, did not give equally good results. It has to be noted however, that, as stated by Anderson (1946), \textit{V. cholerae} is capable of synthesizing some of these substances, such as nicotinic acid and biotin, in the course of cultivation in chemically defined media which originally contained none of these compounds.

\textit{Role of glucose}

As will be gathered from some of the observations recorded above, the incorporation of glucose into the media used for the cultivation of \textit{V. cholerae} was apt not only considerably to promote the multiplication of the organisms but even to render their growth possible under conditions otherwise unsuitable for their propagation. The disadvantage that addition of this sugar to the media is apt to lead in the course of cultivation to a particularly rapid and marked drop of their initial pH is more than compensated by the impetus the presence of glucose gives to the growth of the cholera vibrio.

Hirsch (1928), devoting particular attention to the role of glucose in a valuable study on the metabolism of the \textit{V. cholerae} during aerobic and anaerobic cultivation, established, in addition to the findings already recorded (see section on oxygen requirements, page 112), that, under aerobic as well as under anaerobic conditions, the cholera vibrios by far preferred carbohydrates to amino-acids as sources of energy. Accordingly, the amino-acids (or the peptone) in media which also contained glucose were mainly important as nitrogen sources. As already referred to, Hirsch considered the aerobic growth of \textit{V. cholerae} in glucose-free media as a conditioned process forced upon the organisms by the limitation of the nutritive substances at their disposal. He felt convinced that not this process, but growth under anaerobic conditions in the presence of glucose represented the natural mode of existence of the cholera vibrios in the infected intestine. The present writer, for one, is in full agreement with this contention.

Describing their experiences with the casein digest they originally used without aeration, Jennings & Linton (1944a) concluded

"that glucose serves as an important source of the energy needed for reproduction (of the \textit{V. cholerae}), and that it is utilized in a manner which results in the accumulation of acid in the medium. The organisms are capable of using about 3 grams of glucose per liter before growth is stopped."

However, when growing cholera vibrios in their modified aerated medium, Jennings & Linton (1944b) found that the new cultures could utilize as much as 10 g of glucose per litre, lesser concentrations of the sugar resulting in smaller final crops. Addition of more than 10 g glucose per litre did not lead to an appreciable increase of the yield.
Growth in plain media

While it is proposed to deal with the special media recommended for the rapid isolation or bulk cultivation of *V. cholerae* in a later chapter, it seems indicated at the present juncture to describe the cultural appearances of this organism on plain media.

**Peptone water**

The great value of the method of cultivation or, one should rather say, of enrichment in alkaline peptone water for the laboratory diagnosis of cholera is due to two fundamental properties of the *V. cholerae*, namely, (a) that this organism rapidly grows in the medium, thus out-distancing the contaminating bacteria usually present in the specimens coming for examination, and (b) that the cholera vibrios have a most marked tendency to grow on and near the surface of fluid media. Hence, if one puts a particle of cholera-suspect stools, preferably a mucus flake, or a minute quantity of other materials to be examined into a tube containing 1–2 ml alkaline peptone water with an NaCl concentration of 0.5% (or more up to 3%), incubates the tube for a few hours at 37°C, then takes one loopful of material from the surface of the culture, transfers this into a fresh peptone water tube, and, after further incubation for some hours, takes a loopful from the surface of the subculture and uses this material to inoculate plates containing a suitable solid medium, one may expect, in positive cases, to find on the plates the cholera vibrios in pure culture or at least in a sufficient degree of purity to permit the immediate application of confirmatory tests.

Considerable divergence of opinion exists as to the length of time during which the initial peptone water cultures and the subcultures made from these should be kept in the incubator before material for cultivation on solid media is taken from them. As far as the original publications of the pioneers in this field could be consulted, early workers such as Bujwid (1888), while taking full advantage of serial transfer, incubated their peptone water cultures and subcultures for 24 hours before making use of them. Koch, in an 1893 article on the state of cholera diagnosis postulated in this connexion that

"the best time for examining the peptone solution is 6–12 hours after inoculation, but sometimes one must wait longer. As a matter of fact it is necessary to examine a specimen from time to time, in order to establish the maximal development of the cholera bacteria. Later these are overgrown and replaced by other bacteria even in the upper layers of the fluid, and it may happen that they cannot be demonstrated by too late an examination." [Trans.]
Though shorter periods were considered permissible by some subsequent workers, e.g., three hours by Dunbar (1896) and four hours by Babes (1914), Kolle & Prigge (1928) recommended incubation of the peptone water cultures and subcultures for six hours, while Mackie (1929) advocated a period of six to eight hours. However, in the experience of the present writer it is permissible, during epidemics particularly, to use an incubation period of three hours only, if one takes the precaution of continuing incubation of the peptone water cultures and subcultures, so that material may be taken from them once more, should exceptionally the need arise. Enriched surface-water samples examined with the aid of such shortened incubation yielded analogously satisfactory growth of the cholera-like vibrios abounding in the rivers, ponds, etc., of China.

That the growth of *V. cholerae* in peptone water is rapid indeed has been demonstrated with the aid of biochemical methods by Dunham (1887) and Wakamiya (1940). The former found that weakly alkaline 1% peptone water with an NaCl concentration of 0.5% gave a definite, though slight, cholera-red reaction, if incubated for 4½ hours after inoculation with cholera vibrios. Wakamiya, making comparative test cultivations of *V. cholerae* and *E. coli* in peptone water into which an indicator system had been incorporated, found colour changes in the case of the former organism after 1 hour's incubation, and in the case of *E. coli* after about 3 hours of growth.

The sensitivity of tests with peptone water is well shown by observations quoted by Mackie from a report issued by the British Medical Research Council in 1920, according to which *V. cholerae* could be demonstrated by enrichment in this medium when only four to eight vibrios were present in 25 ml of a dense faecal emulsion.

Since the growth appearances of the cholera vibrio in peptone water are identical with those in nutrient broth, they will be described when dealing with the latter medium. Further reference to the practical use of peptone water for the laboratory diagnosis of cholera will be made in a later chapter.

**Nutrient broth**

As has been noted above, multiplication of the *V. cholerae* in suitable fluid media takes place with such rapidity that it is possible to start subcultivation from them as early as three hours after inoculation. This is all the more remarkable because, as confirmed by the experiences of the present writer, as a rule during the first few hours of incubation no gross evidence of growth becomes manifest in the broth or peptone water tubes or flasks inoculated with cholera materials. It is but gradually, usually after a growth of 12-24 hours at 37°C, that a uniform turbidity develops in such cultures. If one takes care not to shake the tubes, one may sometimes see that at first this turbidity is restricted to the uppermost stratum of the fluids, where the most active growth of the vibrios takes place.
Besides producing a general turbidity in broth or peptone water, growth of *V. cholerae* may, under suitable conditions, also lead even more rapidly to the formation of a pellicle on the surface of these fluid media which, as described by Mackie (1929), is at first semi-transparent and fragile, but gradually becomes thicker and more coherent, and may after an incubation of several days finally sink down in the media.

It was often held that this formation of a surface pellicle was “a phenomenon of surface growth by an organism greedy of oxygen” (Iyengar, 1920), but, as shown by the systematic studies of this worker with broth media prepared with different ingredients and possessing varying degrees of alkalinity or acidity, the presence or absence of a pellicle depended on the one hand of the reaction of the media used, and on the other on the degree of nutrition they afforded to the organisms. Thus in mutton broth prepared by tryptic digestion with an alkalinity above the neutral point of Eyre’s scale (corresponding to a pH of about 8.2), pellicle formation as well as growth of the cholera vibrio were marked, while the slight growth of the organisms in acid mutton broth was not accompanied by formation of a pellicle. The latter remained absent in all broth cultures prepared with beef extract and Witte peptone, in which weak growth of the cholera vibrios took place when the reaction was alkaline, but no growth occurred when the media were acid.

Mackie, besides shortly summarizing the above findings of Iyengar and noting that in the experience of Beauverie (1916) an NaCl concentration of 3% promoted the surface growth of the cholera vibrio, also drew attention to the observation of Wherry (1905) that “the property of pellicle formation could be established by serial transfers from the surface growth, i.e., by a process of artificial selection.” Presumably, this phenomenon was the result of cultural variation which, as will be discussed later, may lead to marked changes in the growth appearances of *V. cholerae* in fluid as well as on solid media.

**Agar**

The colonies of cholera and allied vibrios cultivated on agar plates from fresh materials, such as the dejecta of patients or contaminated water, typically show a rather characteristic appearance, which permits their macroscopic distinction from the often simultaneously developing colonies of contaminating bacteria such as *E. coli*. The vibrio colonies, after 18-24 hours’ incubation at 37°C, appear as regularly circular, pale discs, 1-2 mm in diameter, which show a peculiar bluish lustre (opalescierendes Iriished according to Kolle & Prigge, 1928), when viewed in transparent light. If incubation is continued, the colonies attain a larger diameter (5-7 mm) and may eventually assume a yellowish-brown coloration.
As described by Mackie (1929):

"stroke growth on an agar slope after 24 hours' incubation consists of an abundant, moist, semi-transparent confluent layer which is greyish-white in colour; on continued incubation it becomes more raised and assumes a greyish-yellow tint which deepens after about 10 days to a brown colour".

Gelatin plates

As summarized by Pollitzer (1934b):

"The growth of cholera vibrios on gelatin plates is even more characteristic than that on agar. Though it is necessary to incubate gelatin dishes at the comparatively low temperature of 22°C., one may after 24 hours macroscopically discern colonies represented by very small clear dots. Seen under low power of the microscope they show a peculiarly granulated surface, 'as if strewn with glass particles' (R. Koch). Macroscopically their transparency is in strict contrast to the opaqueness of the bacterial colonies one is apt to encounter when cultivating from faeces. On observing vibrio colonies of recent origin and typical behaviour one notes after about 48 hours a commencing liquefaction of the medium, the colonies appearing to sink into the medium and finally to lie in a small cup or funnel. The process of liquefaction continues, the whole medium becoming dissolved after about 10 days."

While at first glance these peculiar growth phenomena appear to be of great differential diagnostic importance, it has to be realized that they are characteristic of many of the cholera-like as well as of the true cholera vibrios. Moreover, while even freshly isolated cholera strains may show some variation in the rapidity of gelatin liquefaction, the property of liquefying these media may be more or less completely lost by old, often subcultivated, strains. A still greater drawback from the practical point of view is that at the temperatures usually prevailing during cholera outbreaks it is rather difficult to work with gelatin media. It is, therefore, not surprising to find that their use, though much relied upon during the years immediately following the discovery of V. cholerae, has now been given up in favour of other methods of cultivation.

Gelatin stab culture

In gelatin stab cultures, growth occurs along the whole track of the needle but is most marked near the surface where—as a result of liquefaction and evaporation—an air bubble forms within the gelatin. In the past, great stress has been laid upon the diagnostic importance of a typical evolution of this phenomenon. It has now been realized that an important influence is exerted by the composition of the media as well as by the quantity of the inocula used, and that, moreover, cholera-like vibrios may show a behaviour in gelatin stab cultures indistinguishable from that of true cholera vibrios (Pollitzer, 1934b).
Coagulated blood serum

Incubation at 37°C leads to a rapid growth of *V. cholerae* on coagulated blood serum which is initially similar to that on agar. An important difference, however, is that in the case of the former medium liquefaction commences after 24 hours and gradually becomes complete. As aptly stated by Mackie (1929), the property of liquefying coagulated serum, which like that of gelatin liquefaction is due to the proteolytic action of *V. cholerae*, also shows the same range of variability, recently isolated strains differing in the rapidity of liquefaction, while old, often subcultivated strains may more or less fail to produce this phenomenon.

Potato slopes

On potato slopes alkalinized by steaming in 0.7% sodium carbonate solution, fairly abundant growth of *V. cholerae* is usually obtained after incubation at 37°C. In the course of this, chromogenesis becomes frequently marked, so that finally a yellow, greyish-yellow, yellowish-brown or pink coloration results.

As has been noted above, a yellowish-brown coloration also becomes manifest when agar cultures of *V. cholerae* are kept at 37°C for several days.1 Bearing these observations and the experiences with potato slopes in mind, Mackie was disinclined to consider pigment production as a sign characteristic enough to distinguish between the cholera vibrios and certain other vibrios exhibiting marked pigment formation, as had been proposed by Chalmers & Waterfield (1916). As Mackie pointed out with great reason:

"The property of chromogenesis seems a general one in the genus (Vibrio) though more pronounced in certain species according to the medium in which the organism is growing."

Milk

While it is generally agreed that milk is a suitable substrate for the cultivation of *V. cholerae*, markedly divergent opinions have been expressed regarding the growth appearances produced by the organisms in this medium, a few writers even maintaining that in contrast to their usual behaviour the cholera vibrios do not produce an acid reaction in milk media. It has to be noted, however, that the evidence to the contrary brought forward by early workers, such as Kitasato (1889b), Schoffer (1895), Wherry (1905), and Kendall et al. (1914), has been fully confirmed through more recent observations by Pollitzer (1935) and Genevray & Bruneau (1938c). The former worker, carrying out a systematic study at Shanghai, found that

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1 Genevray & Bruneau (1938c), studying about 500 cholera strains isolated during the 1937-38 epidemic in Tonkin, even found that when young agar growths were taken up in large quantity on a platinum loop, a salmon-pink tint became noticeable.
25 cholera strains as well as a larger number of cholera-like vibrios, mostly those isolated from surface waters, invariably produced acidity in litmus milk, usually within 24 hours of incubation at 37°C. Similarly Genevray & Bruneau, studying over 500 cholera strains in Indochina, found that these "invariably turned the colour of litmus milk into pink within 24-48 hours".

While, therefore, there is no valid reason to doubt that cultivation of *V. cholerae* in milk leads to the production of an acid reaction, to what extent this is followed by coagulation of the medium is a rather involved question.

Referring to the initial observations made in this connexion by Koch and his co-workers in India, Gaffky (1887) considered it "most remarkable" that the cholera vibrios, though rapidly and abundantly multiplying in milk, did not produce coagulation or any other macroscopically observable reaction in this medium. However, as summarized by Schoffer (1895), soon afterwards some observers found that the strains at their disposal, which had been mostly isolated in Europe during 1892 and 1893, did coagulate milk. Indeed, this behaviour of the cholera strains derived from the 1892 epidemics in Paris and Hamburg led Liebreich (1893) to the assumption that these outbreaks had been caused by "comma bacilli" different from those isolated by Koch in India.

Though this postulation is now merely of historical interest, it is important to note that, while cholera strains capable of coagulating milk have practically never been met with in India, during the present century, they have been detected upon several occasions in other areas. The following observations may be quoted in this connexion:

(a) Wherry (1905) found that one out of the five cholera strains he had recently obtained in the Philippines was capable of curdling milk within 48 hours.

(b) Examining the strains isolated during the Russian cholera epidemics of 1908-10, Buroff & Buroff (1911) noted that all these growths produced milk coagulation, which became manifest at 37°C on the second day of incubation, at room temperature after 10-12 days. It has to be added that the strains tested by these two workers were also unusual in so far as they proved to be endowed with haemolytic properties.

(c) Working in 1914 with 42 strains which had been isolated during the recent Balkan wars, Popoff-Tcherkasky (1914) found that only five of these growths failed to curdle milk, while the others produced coagulation within 3-11 days. Most of the strains examined by this worker were also haemolytic.

(d) Pollitzer (1935), while finding that the cholera strains isolated in China during cholera epidemics produced no, or only late, coagulation of milk, noted that two out of three strains isolated from sporadic cholera cases at Shanghai in 1934 as well as a strain agglutinable with cholera immune serum which had been isolated at the same time from the Whangpoo River, combined the property of rapidly curdling milk with haemolytic properties. It is important to add that the strains of *V. cholerae* isolated in China during epidemics proved non-haemolytic.

(e) Ali Mustapha (1936), comparing the behaviour of 37 cholera strains with that of 27 El Tor strains, recorded the following interesting results:
BACTERIOLOGY

<table>
<thead>
<tr>
<th>Kind of strains</th>
<th>Number of strains</th>
<th>Behaviour in milk media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains from: India</td>
<td>9</td>
<td>Grew abundantly, producing acidity, but caused with the exception of one Indian strain no coagulation in milk.</td>
</tr>
<tr>
<td>Indo-China</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Strains from minor outbreaks in Baghdad and Bassorah</td>
<td>12</td>
<td>More or less complete coagulation within 2-24 days.</td>
</tr>
<tr>
<td>El Tor strains</td>
<td>27</td>
<td>Massive coagulation, mostly within 24-48 hours, in a minority within 3-15 days.</td>
</tr>
</tbody>
</table>

Mustapha suggested that, since milk coagulation was produced by the El Tor strains, which were as a rule non-pathogenic, and less rapidly also by strains isolated in the Middle East during minor cholera outbreaks, but practically never in India or Indochina, tests with milk media might be a means of distinguishing between cholera strains endowed with marked and low cholerigenic powers, respectively. However, the validity of this assumption is disproved by the observations made in the case of more than 500 Indochina strains by Genevray & Bruneau (1938c), who reported on their experiences with milk media thus:

"All the strains studied change the colour of litmus milk to pink within 24-48 hours and coagulate it. This coagulation commences with the formation of a coagulum 'cap' on the surface of the medium, which often appears within 24-36 hours. The coagulum then slowly grows, reaching a thickness of 2-3 cm within 8 days. If left in the incubator, the whole of the milk becomes coagulated, and the 'cap' more or less shrinks, sometimes undergoing a slight digestion. It has then an aspect similar to that of the soft part of bread." [Trans.]

The question of whether the coagulation of milk by V. cholerae was the result of a rapid formation of acid alone or was partly or even wholly due to the slower action of a rennet-like ferment, which according to Fokker (1892) was produced by the cholera vibrios, has been systematically studied by Schoffer (1895). Making parallel tests with milk samples to which lactic acid alone had been added at varying concentrations and with similar samples in which cholera vibrios were cultivated, he established that the organisms were apt to cause curdling of milk at much lower degrees of acidity than one would have expected from the tests with lactic acid. The action of ferments was, therefore, apparently of paramount, if not of sole importance in the usually slow process of coagulation caused by V. cholerae. At the same time, carrying out seven successive series of tests with different kinds of milk, Schoffer noted a marked inconstancy of the coagulative reactions produced by most of his 14 cholera strains. He raised the question whether these inconstant findings, instead of being

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1 Lamas (1916) had previously established that two El Tor strains were capable of curdling milk within 9 and 20 days respectively, while control strains of V. cholerae failed to do so even within 30 days.
the result of a changing behaviour of the vibrios, were not actually due to changes in the composition of the various kinds of milk used successively, which it was impossible to assess. Schoffer pointed out that this ever-changing character of milk might, to quite some extent, explain the often contradictory statements made regarding the behaviour of \textit{V. cholerae} in this medium. However, while the possible influence of such differences ought not to be disregarded, the available evidence (see also Aida, 1939) seems to indicate, nevertheless, the existence of a parallelism between milk coagulative and haemolytic properties of the vibrios, with the result that the El Tor vibrios are far more prone to curdle milk than the classical non-haemolytic cholera vibrios. It would seem desirable further to elucidate this point by large-scale parallel tests with cholera and El Tor vibrios, particularly in India where thus far but scanty attention seems to have been paid to the behaviour of these organisms in milk media.

\textit{Eggs}

Notwithstanding statements to the contrary made by a few workers such as Hüppe (1888), Kolle & Prigge (1928) maintained that only slight growth of \textit{V. cholerae} takes place in eggs. However, the fact that Wilson (1946), infecting chick-embryos via the allantoic sac with cholera and cholera-like vibrios, noted a multiplication of the organisms not only in the allantoic fluid but also in the amniotic fluid and the egg yolk deserves attention. Moreover, as pointed out by Kolle & Prigge, the egg-broth of Besredka & Jupille (1913) proved an excellent medium for the growth of cholera vibrios as well as for tuberculosis bacilli. Similarly, as will be described in a later chapter, an alkaline egg-peptone medium has been recommended by Goldberger (1914) for selective enrichment in cholera diagnostic work. It also deserves attention that more recently Derkatsch (1927) utilized an alkalized mixture of 150 ml egg-yolk with 850 ml distilled water (pH 7.8) for the differentiation of \textit{V. cholerae} from “para-cholera” and other non-cholera vibrios. He claimed that true cholera vibrios reacted in this medium characteristically by producing within 42 to 72 hours' incubation firm clotting of the substrate, which was followed in 5-7 days by liquefaction with evidence of ammonia production.

\textit{Bile}

It is of interest to add that Ottolenghi (1911) recommended an alkaline bile medium, made with fresh cattle bile, for the enrichment of \textit{V. cholerae} instead of peptone water. Though Ottolenghi’s method was considered useful by some subsequent workers, others, particularly Krombholz & Kulka (1912), found it less reliable than enrichment in peptone water. In the opinion of Kolle & Prigge there was thus no reason to take practical advantage of Ottolenghi’s method. However, as will be further discussed
when dealing with the practical aspects of cholera laboratory work, ample use has been made of bile-salt (sodium taurocholate) agar for the isolation of *V. cholerae*.

**Blood media**

The complex problem of the behaviour of *V. cholerae* in fluid or on solid blood-containing media will be dealt with in a later part of the present chapter.

**Cultural variation**

Kolle & Gotschlich (1903), who were the first to stress the occurrence of variant colonial types of *V. cholerae*, stated in this connexion that

"Petri was the first to point out precisely that in some cultures there occur, besides typical, so-called atypical [colonies], which he called ‘lobated’ colonies. Later observations by Döntz and Pfuhl showed that, if special substances, e.g. asparagine, are added to the nutrient gelatin or if media with a low gelatin content (3%–5%) are used, the cholera colonies do not appear as round, brightly refractory discs with a slightly indented margin, which appear to be bestrewn with very small glass splinters, but show a yellowish coloration, coarser structure and an irregular margin, which sometimes looks frayed (formation of loops), as is common in old laboratory strains, which had been isolated during earlier epidemics.

"A careful examination undertaken in this respect with cultures recently arrived from Egypt in Berlin has left no doubt that in all fresh strains thus received, if they had been subcultivated once or several times, one could invariably find both types of colonies, which we might call transparent and opaque colonies." [Trans.]

Kolle & Gotschlich added that, when subcultivating pure cultures on agar, one also found two types of colonies, those which were homogeneous and others which showed formation of a distinct rim or ring.

As confirmed by the exhaustive studies of Baerthlein (1911a, 1912), the development of variant colonies took place not only on gelatin, but also on agar plates, on which, besides the above-mentioned transparent colonies and ring colonies with an opaque centre and a transparent border, yellowish-white opaque colonies are apt to be present or even preponderant. Baerthlein found that the transparent colonies were composed mainly of slenier, uniformly staining, and well-curved vibrios, whereas microscopic preparations from opaque colonies revealed the presence either of short, thick, bipolar-stained vibrios, or of longer, well-curved forms showing instead of a uniform staining a segmental staining. It has to be noted, however, that in the experience of subsequent observers these morphological differences between the vibrios composing the transparent and opaque colonies respectively were not obligatory.

While finding these two main colonial types remarkably stable if selectively subcultivated at frequent intervals, Baerthlein established that if growths displaying the presence of one of the types only were subcultivated after a lapse of time, once more both colonial types became manifest.
Another point made by this worker was that recently isolated cholera cultures were more prone to show colonial variations than strains kept in the laboratory for a longer time. This seemed to be explained by the observation of Haendel & Woithe (1910) that freshly isolated cholera growths were particularly sensitive to nutritional changes. However, other workers expressed the opinion that cultural variation was "more likely to occur in artificial cultures after continued growth on medium, but may be met with even in newly isolated strains" (Mackie, 1929).

In a later paper, Baerthlein (1918) distinguished between at least nine colonial variants of *V. cholerae* but, as maintained by Gildemeister (1922), most of these seemed to present merely transitory or intermediate forms between the three principal types, the transparent, opaque, and ring forms.

Examining seven cholera strains as well as two El Tor strains and one of "paracholera" vibrios, Balteanu (1926) was able to distinguish between normal, round, translucent colonies and three variants, which he described as follows:

(a) Circumvallate rugose colonies, which were small, opaque, whitish-yellow, and had a thickened margin as well as radially arranged ridges. They were firmly adherent to the medium and could not be satisfactorily emulsified in saline or distilled water. Transferred to broth, these growths produced a thick wrinkled surface film, which broke up on the tube being shaken and sank to the bottom, leaving the liquid clear. Further reference to these rugose colonies will be made in the next section of this chapter.

(b) White ring colonies, which were whitish and semitranslucent and sometimes had an opaque centre and more translucent margin, thus resembling the ring-forms of Baerthlein.

(c) Opaque colonies, which were round, unusually prominent, firm in consistency, adherent to the medium and difficult to emulsify. Transferred to broth or peptone water, these growths produced a thick, hard pellicle, resting on a clear medium, and a large deposit. While this colonial form proved stable on agar subculture, repeated transfers in liquid media led to turbidity and gradual reversion so that, as described by Balteanu:

"Plating from the 10th or 12th daily subculture yielded colonies with translucent margins and after further subcultures colonies of the normal type occurred."

Though Balteanu was able to procure this variant from four of his cholera strains and from one El Tor strain, it was obtained regularly only from one of the former which, as it was found to be haemolytic for human and sheep corpuscles, perhaps ought to have been placed in the El Tor group. Studying this strain alone in detail, Balteanu found, as has been noted earlier in this chapter, that the vibrios composing the opaque colonies possessed no flagella and were, in the opinion of the present writer for this reason, non-motile. Reference has also been made to the presence of a thick mucous envelope round the vibrios. If the flagellar stain recommended
by Yokota (1924) was used, the organisms in question were often uniformly and intensely stained, but less frequently they showed granular or even bipolar staining.

In consideration of these findings it is rather difficult to share the opinion of Balteanu that the unusual colonial form observed by him in an atypical strain was similar to the opaque variant described by Baerthlein. More probably, the appearance of the "opaque" colonies described by Balteanu was due to a process of dissociation, so that they represented the mucoid (M) colonial type. However, in order to decide this point, it would be certainly desirable to make in this respect further studies of the opaque colonies frequently met with in the course of cultivation of typical cholera strains.

The significance of the above described cultural variations of *V. cholerae* has been the subject of considerable debate. Though it has been postulated in some quarters that they were the result of a true, inheritable mutation, there is every reason to share the opinion expressed by Mackie (1929) that these variants "do not, however, represent stable mutants, but are to be regarded as fluctuating variations of the organism".

The observations of several workers that the appearance of colonial variants may be promoted by artificially subjecting the cholera vibrios to unfavourable influences, e.g., to free chlorine or phenol (Genevray 1940a, 1940b, 1940c), serve as a corollary for the assumption that such variations are the result of a temporary adaptation and not of a permanent mutation.

It is, however, noteworthy that among the four types of cholera colonies distinguished by Husain & Burrows (1956) by cultivation on thionin-glycerol agar only the "granular chromatic" type occurred on the plates used for the primary isolation of the causative organisms from the patients' stools. Hence it seemed probable to the two authors "that colonial types other than this may be regarded tentatively as variant types of uncertain relation to the human disease".

It is important to note that colonial variation has been demonstrated not only in the case of the classical *V. cholerae*, but also in the case of the El Tor vibrio (Balteanu, 1926; Alessi, 1939) and, as has been shown for instance by Feldmann (1917) and by Pasricha, De Monte & Gupta (1932), likewise in the case of cholera-like strains.

**Dissociation**

Indispensable though it is to refer to the phenomena of dissociation at the present juncture, these processes, being apt to exert a profound influence on the immunological properties of the *V. cholerae*, cannot now be fully appreciated. More than that, as shown, for instance, by recent observations on the dissociation of *V. cholerae* by Bhaskaran (1953), a change in the growth appearances of this organism is by no means indispensable for indicating the presence of dissociation, which may often be demonstrated by biochemical or serological methods in cultures presenting no atypical
features as far as their macroscopic aspect is concerned. On the other hand, it is not surprising to find that, though the presence of macroscopically characteristic dissociants had been noted by some early workers, the occurrence of such atypical colonies was confused with that of colonial variants which, as has been described, resulted from an adaptation of the cholera vibrios to unfavourable environmental conditions.

Hadley (1927), dealing comprehensively with the early observations of dissociation in the various bacterial species, mentioned a few records dating back to 1894 which, in his opinion, referred to dissociants of *V. cholerae*. It would seem, however, that Berestneff (1908) was the first who definitely noted the rough form of this organism, stating:

"that cholera vibrios, if repeatedly transferred from agar to agar, sometimes begin to grow in the form of dry, prominent and non-confluent colonies. Many such colonies show a crater-like depression and a wall-like periphery. Such colonies are markedly different from the normal ones; on account of their dryness they are difficult to emulsify and show pseudo-agglutination, being thus unfit for agglutination tests."

Though there can be little doubt that Berestneff referred to the rough form of *V. cholerae*, it was only after the pioneer studies on microbial dissociation had been published by Arkwright (1921) and De Kruif (1921) that Shousha (1924) gave a full description of the properties shown by the smooth (S) and rough (R) types of this organism respectively.

Shousha worked with two old cholera laboratory strains, one of which proved to be haemolytic when tested with sheep erythrocytes. Both were inoculated into broth tubes which, after an incubation at 37°C for 24 hours, were stored at room temperature in the dark. When agar subcultures were made after such storage for 15 days, the haemolytic strain only showed two types of colonies similar to the S and R forms described by Arkwright (1921) in salmonellae.

While not referring to the morphological appearances of the vibrios composing these two types of colonies respectively, Shousha stated that both were equally motile. The differences in growth appearances observed by him may thus be summarized:

<table>
<thead>
<tr>
<th>Medium</th>
<th>S type</th>
<th>R type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>Colonies circular with well-defined margins, finely granular under low power of the microscope.</td>
<td>Colonies larger, flat and thin, appear granular with jagged margins when seen under low power of the microscope.</td>
</tr>
<tr>
<td>Broth or peptone water</td>
<td>General turbidity and pellicle formation.</td>
<td>Deposit at bottom and pellicle formation, the body of the fluid remaining clear.*</td>
</tr>
</tbody>
</table>

* Uniformly turbid growth was obtained either if media prepared with less salt were used or if the usual media were diluted to one-half or one-quarter with distilled water.
Since the publication of this initial study, the phenomena of S-R dissociation of *V. cholerae* have been exhaustively examined by different observers. The following of their findings have a bearing on the problems now under review.

Referring to the morphological appearances of the vibrios from smooth and rough cholera colonies respectively, Seal (1937) maintained that

"the individual cell of the rough type has a more opaque and granular cytoplasm and a thicker outline than that of the smooth one which, on the other hand, possesses a clear cytoplasm and a thin wall. This may explain why the rough colonies usually look opaque and the smooth ones clear and translucent to the naked eye."

Panja (1945), who was able to produce rough colonies of *V. cholerae* by subcultivating smooth strains on agar into which mepacrine had been incorporated in the proportion of 1:5000, noted that in the case of the Inaba subtype some of the vibrios composing the rough colonies showed straight, spherical, or ovoid forms and were sometimes immotile. Ogawa rough vibrios showed long, straight, besides typically curved forms, but were invariably motile.

Further observations on the morphological differences between vibrios composing rough and smooth colonies respectively were recorded by Wahba (1953). In his experience "unlike other organisms, the cholera vibrios in the rough state do not always appear in long or filamentous forms; on the contrary they might even be shorter and stouter than in the smooth state." Making impression films from well separated colonies, Wahba found the rough colonies to be composed of three zones: a central irregularly arranged core, an intermediate zone composed of radially lying organisms, and peripherally outgrowing tufts composed of filamentous organisms. However, the outer zone was not filamentous in the case of some rough strains. Smooth cholera colonies appeared to possess two zones only, both of which were composed of radially arranged organisms.

Marked differences existing between smooth and rough cholera vibrios in regard to the mode of cell division and colony formation were described by Seal (1937) thus:

"The essential difference depends upon the degree to which the contiguous cells adhere to each other after undergoing division. The final cluster in a smooth culture is even in appearance owing to the cells sliding past each other and forming a smooth and compact mass, while in a rough culture the tendency to slip past each other is almost absent and the cells after division tend to adhere to each other more firmly leading to the formation of bending and branching chains and irregular masses with many open spaces, projections, angles and sometimes chains, sticking out from their sides, the final cluster being thus jagged and uneven in appearance."

The differences existing between smooth and rough growths of the *V. cholerae* in fluid media were in Seal’s opinion due to identical causes. In the case of the smooth type, cultivation in broth or peptone water
resulted in a marked and uniform turbidity with or without a thin pellicle, because the vibrios did not tend to stick together after division. The rough strains, on the contrary, formed chains and the irregular clusters thus resulting led to the formation of a thick pellicle on the surface of the fluids as well as to the formation of granules, which as a rule sank down but could remain partially suspended, then producing a slight turbidity of the media.

Observations made by Soru (1934) with 106 cholera and cholera-like vibrios showed that vibrios of the R type had a higher negative electric charge than those of the S type. These results were confirmed by Linton, Mitra & Seal (1938), who partly examined dissociants they had obtained from Bruce White. Linton and co-authors noted the interesting fact

"that electrophoretically the organisms which are quite distinct from one another in the S state are often similar or identical in the R state. This is perhaps the underlying factor to account for the observation of Bruce White who found 'R'-strains serologically more generalized than the 'S'-strains. In the case of Shillong 1077, the ρ strain showed an even higher surface potential than the rough homologue and was very much higher than the original smooth strain."

The studies of Bruce White referred to above led to a full understanding of the phenomena underlying dissociation of the V. cholerae by furnishing evidence to prove the contention made in a preliminary statement by Yang & White (1934) that in case of V. cholerae as in that of the salmonellae and the pneumococci "roughening involves the disappearance of a non-protein and probably carbohydrate containing substance which furnishes the characteristic O-receptor of the smooth organism". "It seems certain," Yang & White continued, "that a second non-protein ... substance, present but masked in the smooth organism, replaces in the rough vibrio the lost smooth factor and becomes the characteristic rough receptor."

In a further paper published in 1934, Bruce White established that in the case of V. cholerae as well as in that of the salmonella group a ρ variant existed, which differed from the R form by loss of the dominant R receptors. Bruce White added that according to various tests

"this loss involves the bulk of those receptors which are supplied by the alkali-resistant, non-protein and richly carbohydrate soluble substance of the rough vibrio."

Continuing his studies, Bruce White (1936) was able to establish that "in each strain of V. cholerae and seemingly in vibrios in general, at least four distinct groups of polysaccharide receptors or substances are concerned in the serology of the normal parent and variant forms". All four of these substances, named Cα, Cβ, Cγ, and Cδ, were present in the smooth form, but Cα was dominant. In the rough form, Cα was absent and Cβ was dominant, whereas only Cγ and Cδ were present, and the latter was dominant, in the ρ form. The distribution of these four polysaccharides may, therefore, be schematized thus:
Bruce White added that, though no variant degraded below the level of the \( p \) form had been discovered, such forms possibly existed. Carrying out comparative studies he found that

"the true El Tor vibrio presents a polysaccharide complex serologically identical with that of \( V.\ cholerae \) (of the same absorption type); that the \( C_\gamma \) and \( C_\delta \) factors seem to be common, so far as can be judged by simple precipitation tests, to all the types of vibrios so far examined; that different groups of vibrios show sharp differences in the behaviour of their \( C_\beta \) substances; and that the \( C_\alpha \) substances determine the serological specificity of the various smooth types."

Besides elucidating the phenomena of S-R dissociation of \( V.\ cholerae \), Bruce White (1938, 1940) also dealt in a masterly manner with the rugose form of this organism. He stated in the latter connexion that Balteanu (1926), working with cholera and El Tor vibrios, had noted the occurrence of a variant colonial form which he designated as rugose on account of its corrugated appearance on agar. However, Bruce White added:

"From a detailed description of the rugose variant Balteanu was probably deflected by the fact that it proved unstable in culture: his attention was occupied with a more stable 'opaque' variant which is perhaps a cultural form much of the same genre."

This is in accordance with what has been stated above in regard to Balteanu’s "opaque" form.

Even though, as shown in Table XVIII, the appearances and properties of rugose growths are markedly different from those of the rough forms of \( V.\ cholerae \), some workers were inclined to regard the rugose variant as the extreme type of roughness. It is the great merit of Bruce White to have pointed out that actually a fundamental difference exists between the two modes of dissociation concerned: while, as described above, the process of roughening is due to a failure to secrete or to form specific polysaccharides, rugosity is the result of an abnormally active secretion of a mucinous material, ascribed by Bruce White to an intensification of normal secretory processes rather than to a special type of activity. In fact, a transition to the rugose state could be observed in the case of rough or even \( p \) growths as well as in smooth growths of cholera vibrios and particularly of El Tor vibrios. The marked tendency of the rugose derivatives to return to their original state (\( S, R, \) or \( p \)), as contrasted with the stability of the ordinary \( R \) form, also rendered it altogether improbable that rugosity represented a culmination of roughness.

Though observed in growths from cholera stools, the rugose type of colonies was particularly met with in platings from aging peptone water
TABLE XVIII. CHARACTERISTICS OF S, R AND RUGOSE GROWTHS OF V. CHOLERAE *

<table>
<thead>
<tr>
<th></th>
<th>Normal S culture</th>
<th>Typical R culture</th>
<th>Rugose culture derived from S culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance of colonies on agar plates.</td>
<td>Circular, moderately raised, clear and moist; may be granular under lens and of variable transparence or turbidity. Occasionally, colonies may show features usually associated with roughness.</td>
<td>As a rule differs but slightly in general appearance from S colony, so that it cannot be identified with certainty by simple inspection. Usually clearer of surface and more coarsely granular than S colony. Rugose culture and flattening. Outward appearance, not indicative of intensity of appearance through focus.</td>
<td>The 18 hours' colony small, much raised and retractile. It increases rapidly with further incubation and develops superficial corrugation, irregular, radial, or both. Opaque yellowish or yellow in colour, opacity and tint deepening with age. In older colonies vibrioid or granular &quot;corona&quot; may be exuded from margin of the colony.</td>
</tr>
<tr>
<td>Consistence and adherence.</td>
<td>Slightly fluid, never adherent to medium.</td>
<td>Dry, brittle, never adherent.</td>
<td>Tough or gelatinous, adherent to medium.</td>
</tr>
<tr>
<td>Dispersibility and agglutination in NaCl solution.</td>
<td>Disperses readily with persistence some initial sliminess. Dense suspensions in 1% NaCl solution may show some precipitation or slime enveloping a few vibrios.</td>
<td>Disperses readily in casein or 1% NaCl solution but complete agglutination follows slowly, so that culture rests in clear liquid.</td>
<td>Growth disperses only partially and with difficulty. Vibrios once dispersed are insensitive to saline.</td>
</tr>
</tbody>
</table>

* After Bruce White (1938)

...cultures, and it appeared that higher peptone concentrations (5%-10%) favoured this mode of growth. More important still, rugose colonies were found to abound in platings of vibrios which had survived specific bactericidal tests.

Evaluating these observations, Bruce White (1940) stated:

"It is difficult to escape the conclusion that the rugose substance is a protective secretion with a role in assisting the survival of the race in nature. In the laboratory it affords defense against unfavourable conditions and the action of serum: it has repeatedly been observed that rugose forms tend to grow often in pure culture, from mixtures made in specific bactericidal tests and to survive their associates in ageing cultures of vibrios."

The rugose variants did not, however, prove more resistant than normal cholera vibrios when kept in saline solutions or grown in broth to which hydrochloric acid had been added, so that there was no reason to assume that they would have a particularly good chance of resisting the acid conditions in the human stomach. Nevertheless, the evidence that they are more resistant than non-rugose vibrios is convincing. Considering this, as well as the marked tendency of rugose growths to revert to type, one might look upon rugose transition as a means of prolonging the life of infective cholera...
strains whereas, as will be discussed later, the stable degradation brought about by transition into the rough state is instrumental in rendering the vibrios concerned non-infective.

Though the occurrence of a third type of dissociation of *V. cholerae*, leading to the growth in the form of minute pleuropneumonia-like (L) colonies, has been established through recent observations only (Minck, 1950, 1951; Minck & Minck, 1951; Carrère & Roux, 1953), the presence of dwarf colonies has been recorded by some earlier workers, first apparently by Baerthlein & Grünbaum (1916).

These two workers stated that some diagnostic difficulties were caused by the occurrence of minute colonies, reaching hardly pin-point size within 24 hours of incubation even on Diendonné-agar, which often developed alone on the plates used for isolation of *V. cholerae*. Smears from such growths showed the presence of very slender vibrios which, as they formed chains, resembled relapsing fever spirochaetes. Subcultivation on solid media did not lead to a change in the growth appearance of such strains. However, if the vibrios were kept for some time in broth and then subcultured on suitable media, typical, opaque and ring colonies developed.

Minck & Minck (1951—see also Minck, 1950, 1951) were able to produce L-dissociation of *V. cholerae* through subcultivation of primary cultures from intraperitoneally infected mice on soft serum agar containing 1000 units of penicillin per ml, but had no success with stock cultures. After an incubation of the penicillin-containing cultures for six to eight hours, dwarf colonies with a maximal diameter of 500 μ became visible. Their centre was found to consist mainly of minute L forms ("elementary bodies"), while on the periphery giant globular bodies (diameter 10-20 μ) were seen which were more or less filled with motile granules. Intermediate forms were likewise encountered.

It was possible to maintain the strains in this dissociated condition by weekly subculture on penicillin-serum agar. Transfers from these subcultures to ordinary serum-agar led at first to the development of normal cholera colonies, but after five to six passages through penicillin-containing media, dwarf colonies developed even on ordinary serum-agar and proved stable upon subcultivation on the latter medium.

Intraperitoneal inoculation of mice with little-subcultivated L growths led to no pathological changes but seemed to confer a certain degree of immunity against infection with normal cholera vibrios. Inoculation with "fixed" L growths (i.e., those showing no tendency to revert to type on ordinary serum-agar) often led to the death of the animals. Autopsy showed the presence of acute peritonitis and necrotic enteritis. On two occasions only, a few L colonies developed in cultures from the peritoneal exudate, while, as a rule, enteric bacteria alone seemed to be present.

The findings of Carrère & Roux (1953) were on the whole similar to those described above. It is noteworthy, however, that according to their observations (a) a stock Inaba strain of *V. cholerae* was found to produce numerous L forms and globular bodies in ordinary broth; (b) L forms developed on semi-solid media inoculated with the filtrate of a five-day-old dissociant peptone water culture through an L3 Chamberland candle; and (c) the L forms appeared to be non-pathogenic for white mice, while percutaneous or subcutaneous as well as intraperitoneal inoculations with
material containing globular bodies killed these mice in 24 hours. Carrère & Roux were inclined to assume that the globular bodies might be a resistant form of *V. cholerae*.

### Biochemical Properties

#### Specific chemical constituents

Though Galeotti (1912) claimed to have isolated a nucleoprotein of *V. cholerae* as early as in 1896, and Landsteiner & Levine (1926) laid a firm foundation for future work by extracting from a cholera strain a carbohydrate-containing substance which reacted specifically with cholera-immune serum, it was only within the last two decades that systematic studies on the immunochemistry of the cholera vibrio have been carried out by Linton and his co-workers as well as by some other investigators, particularly Bruce White, whose observations will, however, be considered in the following chapter.

Linton and his co-workers (see Linton, Shrivastava & Mitra, 1935; Linton, 1940, 1942), observing the optical activity of the proteins of numerous cholera and cholera-like strains in dilute alkali solutions, were able to distinguish between two types of protein. They also found that three types of polysaccharides occurred in these vibrios, composed respectively of (1) galactose and an aldobionic acid consisting of galactose and glycuronic acid; (2) arabinose and an aldobionic acid identical with that of group (1); (3) glucose only. It was thus possible to class the cholera and cholera-like vibrios into six groups composed as follows:

<table>
<thead>
<tr>
<th>Number</th>
<th>Protein type</th>
<th>Polysaccharide type</th>
<th>Nature of vibrios composing group</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>I</td>
<td>I</td>
<td>Majority of true cholera strains, occasionally water vibrios.</td>
</tr>
<tr>
<td>II</td>
<td>I</td>
<td>II</td>
<td>Cholera strains, specially those from Assam (rare in Calcutta), occasionally water vibrios.</td>
</tr>
<tr>
<td>III</td>
<td>II</td>
<td>II</td>
<td>Mainly water vibrios, not agglutinable with cholera immune serum.</td>
</tr>
<tr>
<td>IV</td>
<td>II</td>
<td>I</td>
<td>El Tor strains and some identical strains isolated from carriers in India.</td>
</tr>
<tr>
<td>V</td>
<td>II</td>
<td>III</td>
<td>Cholera strains from carriers.</td>
</tr>
<tr>
<td>VI</td>
<td>I</td>
<td>III</td>
<td>Rare in nature, mainly found in old laboratory strains of <em>V. cholerae</em>.</td>
</tr>
</tbody>
</table>

As will be noted, even apart from the tediousness of the procedures involved, it would not be possible to make with the aid of these tests a diagnostically valid distinction between cholera and cholera-like vibrios. It is, however, interesting to see that the El Tor vibrios, though showing in most respects features identical with those of the classical non haemolytic cholera vibrios, fell according to the above method of classification into a separate group.
Linton, Mitra & Mullick (1936), studying the respiration and glycolysis of cholera and cholera-like vibrios, found metabolism to be most active when the organisms belonged to Group I, less so in the case of Groups II, V and VI, least active in case of the Group III vibrios. Group IV, to which the El Tor vibrios belonged, was according to these workers "sharply marked off" by the absence of glycolysis under aerobic conditions of growth. Rough dissociants showed a lower metabolism than their smooth parent strains.

The observation that vibrios of Group I, to which most true cholera vibrios belonged, was metabolically most active, is in accord with Bernheim's observation (1943) that *V. cholerae* had 24% more reactive amino groups than *E. coli*. Since then, the presence of hitherto unknown amino-acids of the cholera vibrio has been recorded (Blass & Macheboeuf, 1945, 1947; Blass et al., 1951; Banerjee, Roy & Ganguli, 1956; Blass, 1956).

**Enzymatic make-up**

**Proteolytic enzymes**

As far as could be ascertained, Bitter (1886) was the first to establish that the liquefaction of gelatin-containing media by *V. cholerae* was due to the presence of a proteolytic enzyme or, as he called it, "ferment" which exerted an influence analogous to that of trypsin. Wherry (1905), summarizing further early observations made in this direction, stated that, in analogy with the behaviour of trypsin, the proteolytic enzymes of the cholera vibrio as well as of other bacteria were operative only in alkaline media, the presence of even small amounts of acids hindering their action. The presence of fermentable carbohydrates in the media was found to inhibit the liquefaction of gelatin, but it deserves attention that according to Auerbach (1897) who, though devoting some attention to *V. cholerae*, experimented mainly with *Proteus vulgaris*, this absence of liquefaction was due not to the appearance of acids in the course of cultivation but to an inhibition of the formation of the proteolytic enzymes. Besides the presence of protein substances in the media, the access of free oxygen was found to be essential for the production of these enzymes, liquefaction of gelatin taking place very slowly, if at all, under anaerobic conditions (Liboriuss, 1886).

Summing up his own observations, Wherry stated that the type of liquefaction was influenced to a marked degree by the melting point as well as the reaction of the gelatin used, and added that

"the optimum condition for growth is furnished by an albuminous medium containing between 1/50 and 1/100 gram-molecule of NaOH or Na₂CO₃ per liter, and this corresponds fairly well with the optimum conditions for the tryptic digest of fibrin".

In a recently published study, Agarwala & Shrivastava (1953) recorded the results of viscosimeter measurements of the "gelatinase" activity of
cholera and cholera-like strains grown for 24 hours in papain broth to which gelatin at a concentration of 5% had been added. They found that growths of cholera-like water vibrios displayed a 25% greater gelatinase activity than all other strains tested, which included, besides true cholera vibrios, also those of the El Tor group. The pH optimum for the gelatinase activity of both true cholera and water vibrios was found to be about 8.0. Incubation of the cultures for longer periods failed to increase the activity of the enzyme which was found to be stable for a long time in growths kept at 37°C.

As recently stated by Nihoul (1952), the presence of calcium exerted an impeding effect on the proteolytic activity of V. cholerae.

While, as apparently first demonstrated by Beaujean (1913) and generally accepted, no correlation exists between the proteolytic and the haemolytic properties of the cholera vibrios, the question of the relationship between the former property and the haemodigestive action of these organisms, referred to later in this chapter, has been the subject of debate. Bernard, Guillerm & Gallut (1937a, 1937b) reached the conclusion possibly arrived at earlier by Loewy (1915) that the proteolytic and haemodigestive properties of the V. cholerae were due to the action of one and the same enzyme, but Beeuwkes (1939) adduced evidence to show that probably different enzymes were responsible for the gelatin liquefaction and haemodigestion respectively produced by cholera and El Tor vibrios.

Milk-coagulating enzyme

As has been discussed above (see page 123), the production by V. cholerae of an enzyme identical in action to that of rennet, which was capable of coagulating milk even in the presence of a weakly acid reaction, has been demonstrated by Fokker (1892).

Collagenase

Studying the action of culture filtrates of V. cholerae on pure collagen prepared from buffalo tendons, Narayanan & Menon (1952) stated that they had demonstrated the presence of a collagenase. This enzyme, which was found capable of acting over a wide pH range with an optimum at pH 8.0, was probably also present to some extent in the culture filtrates of cholera-like vibrios, but could not be demonstrated in those of E. coli.

Elastinase

In a further study published in 1953, Narayanan, Devi & Menon reported on the presence of an elastinase, active against elastin prepared from buffalo ligaments, in the cultures of two out of the 7 cholera strains examined in this respect. The presence of this enzyme as well as that of collagenase was also demonstrated in cultures of cholera-like vibrios.
Lecithinase

Reporting in 1944 on the lecithinase activity of *V. cholerae*, Felsenfeld referred to earlier studies made in this respect by Ruata & Caneva (1901) and Kraaij & Wolff (1923). While the latter workers demonstrated the presence of lecithinase only in El Tor vibrios, Ruata & Caneva found this enzyme to be present in all vibrio strains examined by them. Felsenfeld's investigations also showed lecithinase activity in four true cholera strains as well as in one El Tor strain. The optimal temperature for the action of the enzyme was 36°-38°C, the optimal pH 7.4-7.6. Lecithinase activity was stimulated by calcium and magnesium, whereas formaldehyde, phenol, and fat-soluble narcotics exerted an inhibitory effect.

Deaminases

As recorded by Dudani et al. in 1952 (see also Iyer et al., 1953, 1954; Iyer & Krishna Murti, 1955), *V. cholerae* possess deaminases in their enzyme make-up, the rate of deamination varying from one amino-acid to another, and differing in different strains. Among the amino-acids studied, deamination of aspartic acid and serine was maximal, but arginine, glycine, glutamic acid, lysin, and threonine were also deaminated. Deamination took place under strictly aerobic conditions only and was optimal at a pH range of 7.0 to 8.0. It is interesting that in general cholera vibrios of the Ogawa subtype showed a higher deaminase activity than those of the Inaba subtype.

Further investigations by Arora and colleagues (1956) showed that sodium chloride exerted a stimulating action on the production of certain deaminases by the cholera vibrio.

Studying the metabolism of purine and pyrimidine compounds of a few strains of *V. cholerae* and other vibrios, Agarwala et al. (1954) found that deamination appeared to be the only active process in the utilization of purine nitrogen.

Nucleotidase

According to observations by Krishna Murti & Shrivastava (1955), *V. cholerae* and cholera-like vibrios, while possessing no phosphatase activity, were endowed with nucleotidase activity and were thus capable of readily attacking the phosphoric acid ester linkage of purine and pyrimidine compounds.

Dehydrogenases

Dudani et al. in 1953 published the results of preliminary observations on the dehydrogenation of various substrates by an Ogawa strain of *V. cholerae* and the Inaba and rough variants derived from it. Almost all amino-acids and aliphatic acids employed in this study were found to
be capable of acting as hydrogen donors for the respiratory activity of the organism. The dehydrogenases of *V. cholerae* seemed to be linked up with the cytochrome systems present in this organism, but the possibility of other coenzyme systems taking a part in the process of dehydrogenation could not be excluded.

*y*-peptidase. As stated in a preliminary report, Agarwala et al. (1953b), studying the hydrolysis of glutathione by *V. cholerae*, found evidence pointing to the probable presence of *y*-peptidase in the cells of this organism.

*Mucinase and tissue-disintegrating enzyme*

The important studies of Burnet (1948, 1949) and Burnet and co-workers (1946, 1947) on the enzymes of *V. cholerae* go back to investigations made by Burnet, McCrea & Stone in 1946 on the receptors of human red blood corpuscles for virus action. These workers found that red cells which had been treated with influenza virus, while losing their agglutinability by some or all of the viruses of the mumps-influenza group, developed an agglutinability with almost any human serum (“pan-agglutinability” of Thomsen, 1926). Since Friedenreich (1928) had shown that such a pan-agglutinability with sera of all blood groups and even with that of the donor of the red cells could be produced with the aid of cholera and cholera-like vibrios, Burnet, McCrea & Stone experimented with the culture filtrates of two cholera and one cholera-like strain. They found that an action almost completely analogous to that of the above-mentioned viruses, and due undoubtedly to enzyme activities, could be produced with these culture filtrates.

Following up this work, Burnet & Stone (1947) made tests with various substrates to explore the possibility that the receptor-destroying enzyme of the filtrates of cholera cultures was a collagenase. The important result of these studies was the demonstration of an actively desquamating effect exerted by these filtrates on the intestinal mucosa of guinea-pigs and rabbits. As stated by Burnet & Stone,

“it soon became evident that this action was not a function of the receptor-destroying enzyme but the possible relationship of such *in vitro* desquamation to the pathogenesis of human cholera seemed to justify an independent investigation of the phenomenon”.

Burnet & Stone summarized the results of this investigation thus:

“(a) Filtrates from *Vibrio cholerae* cultures are capable of producing desquamation of the intestinal epithelium *in vitro*;

“(b) There is a well-marked gradient of diminishing susceptibility to desquamation from the jejunum to the descending colon;

“(c) Histological and other preliminary evidence suggests that the principal agent concerned is a mucinase;

“(d) Intestinal mucin is rapidly dissolved by active filtrates, the effect paralleling the desquamation reaction; both are similarly neutralized by rabbit immune serum (prepared with the aid of *V. cholerae* culture filtrates);

“(e) Evidence is given for the existence of another enzyme concerned with breaking down the cement substance between cells.”
Discussing the importance of these findings, Burnet & Stone pointed out that

"the mucinase described in this paper can rapidly destroy the viscosity and hence the mechanical protective and lubricating properties of intestinal mucus. Experiments in progress show that this action can take place in isolated gut segments in the living animal and if the enzyme, as seems likely, is produced in large amount in the bowel of a cholera patient, it might well play a major part in facilitating desquamation of the intestinal epithelium. The third agent (i.e. the tissue-disintegrating enzyme), on which very little work has so far been done, by breaking down some presumed components of the cement substance between cells would also favor the desquamating process."

No evidence was obtained to show that the receptor-destroying enzyme played a role in this process of desquamation and tissue-disintegration.

Reporting on further studies of the cholera mucinase Burnet (1948) stated that this enzyme was found to be active against a variety of glandular mucins but exerted no action on human synovial fluids (hyaluronic-acid-type mucin). The activity of mucinase was found to be completely inhibited by sodium hexametaphosphate and (like hyaluronidases) it was inert in the absence of salts.

As recorded by Burnet in a further communication published in 1949, it had been found possible to treat the vibrio filtrates so that they contained either mucinase or the receptor-destroying enzyme alone in active form: if the filtrates were treated with an excess of CaCl₂, brought to a pH of 6, and heated for 30 minutes at 56°C, the mucinase alone was destroyed. However, if the filtrates were alkalinized to pH 8.5 and held for some hours at 37°C, the mucinase remained fully active while the receptor-destroying enzyme was totally inactivated.

Publishing further observations on the intestinal-epithelium-destroying enzyme Singh & Ahuja (1953) stated that they could demonstrate its presence not only in all smooth cholera strains but also in most El Tor and cholera-like strains examined by them. These two workers concluded, therefore, "that mucinase activity is not specifically confined to V. cholerae but is shared by other members of the genus vibrio. Whether or not this enzyme plays any role in the causation of [the] cholera syndrome is a moot point."

Making a study of the serological character of the mucinase of V. cholerae and other vibrios, Freter (1955b) established in analogy with the views of Singh & Ahuja that three strains of "non-agglutinating" water vibrios isolated in Chicago produced high-titre mucinases which in two cases were serologically related to the enzymes of the cholera vibrios. However, while maintaining that apparently "the titer and serological type of mucinase produced in vitro has no relation to virulence, colonial morphology or O antigenic structure of the tested cholera strains", Freter was careful to point out that "the data presented do, of course, not give information as to the actual relation of mucinase or other enzymes to the pathogenicity of cholera vibrios".
In regard to the latter point it deserves attention that (1) Freter (1955a) was able to demonstrate mucinase in the bowel fluid of guinea-pigs which had succumbed to enteric cholera infection; and (2) Lam, Mandle, & Goodner (1955) adduced experimental evidence that

"V. comma mucinase (or the mucinase complex of enzymes) alters the permeability of the mouse intestine but that this effect is blocked by immunization against mucinase both by passive and active procedures."

**Penicillinase**

Investigations by Iyer, Dudani & Krishna Murti (1954) indicated that cholera and El Tor vibrios did not exhibit any penicillinase activity and that consequently the low susceptibility of V. cholerae to penicillin could not be due to the elaboration of such an enzyme by the organisms. Four among the nine strains of cholera-like vibrios tested were found to produce penicillinase.

**Decarboxylase**

According to Ogasawara & Kariya (1954), cholera vibrios were capable of producing at an acid pH a lysine decarboxylase enzyme which converted lysine to cadaverine (pentamethylene diamine). The action of this enzyme probably accounted for the presence of cadaverine in the stools of cholera patients.

**Lipase**

The production of a lipase active against olive oil by V. cholerae as well as by cholera-like vibrios has been recently demonstrated by Narayanan, Devi & Menon (1953).

**Carbohydrate-converting enzymes**

Bitter (1886) seems to have first drawn attention to the amylolytic activity of the V. cholerae due to the action of "ferments" (enzymes). His observations were soon confirmed by several other workers (see Nobechi, 1925). Wherry (1905), one of the pioneers in this field, stated that all six cholera strains examined by him produced not only amylase and maltase (as had been previously found to be the case by Buxton, 1903) as well as invertase (already found by Sclavo, 1892), but also lactase.

**Indole formation**

As will be described in a later chapter, application of Ehrlich’s rosindole test shows that cholera vibrios, if suitably cultivated, invariably produce indole. Since, however, other intestinal bacteria as well as many of the cholera-like vibrio species also react positively in this respect, such tests have no differential diagnostic importance.
As summarized by Sticker (1912), Hoppe-Seyler (1892) found that indole accumulates in the intestine of cholera patients because it is no longer destroyed by oxidation as in the healthy body. The large amounts of indican and indoxyl sulfuric acid found in the urine of cholera patients also indicated according to Hoppe-Seyler an increased indole production.

**Nitroso-indole (cholera-red) reaction**

It is curious to note that tests based on the phenomena underlying the nitroso-indole or, as it is commonly called, the cholera-red reaction, carried out after 1883 with cholera cultures, had been utilized well before that year with the aid of the dejecta of cholera patients. According to Sticker (1912), Kopp (1837) was the first to observe that addition of small amounts of pure nitric acid to cholera stools or their distillates produced a red colour, and similar results were recorded by some subsequent observers including Virchow (see Schuchardt, 1887), who partly used other mineral acids.

After Koch had isolated *V. cholerae*, the testing of suspicious cultures with mineral acids, so as to determine whether or not the red coloration considered characteristic for this organism appeared, was recommended by Poehl (1886) and independently by Bujwid (1887), and Dunham (1887). Bujwid emphasized in his short note, which appeared before Dunham’s article, the importance of the method for a rapid diagnosis of cholera, since a pink to reddish-violet colour appeared quickly when a few drops of 5% to 10% hydrochloric acid had been added to broth cultures of cholera vibrios grown at 37°C for 10-12 hours. He considered the test as practically specific for *V. cholerae*.

Recording the results of an investigation into the phenomena underlying this test, Salkowski (1887) stated with admirable clearness and brevity that the “cholera reaction” is

“nothing else than a quite common indole reaction, and the explanation for the fact that the indole reaction can be produced in cholera cultures with sulfuric acid alone is simply that the cholera vibrios constantly produce nitrous acid, which is present in the fluid in the form of nitrites. There exists no specific cholera red, as has been assumed by Brieger; this is simple indole-red and demonstrable in every decomposing peptone solution. Characteristic of the cholera bacteria is only the simultaneous production of indole and nitrous acid.” [Trans.]

The validity of Salkowski’s statement that the cholera-red reaction is due to the ability of the cholera vibrio of reducing nitrates to nitrites as well as to the production of indole by this organism has been generally accepted.

The technique of the nitroso-indole test will be duly described in a later chapter. It has to be noted, however, that, whereas the early workers considered this reaction one of the principal methods, or even the cardinal method of establishing the presence of *V. cholerae*, it has now hardly any importance in practical cholera laboratory work. For it has been
established that on the one hand positive reactions are also produced by certain cholera-like vibrios and even by bacteria belonging to other genera, while on the other hand, for reasons which will be specified when dealing with the problems of cholera diagnosis, false negatives may be obtained even though *V. cholerae* is present. However, as will be noted later, Taylor, Pandit & Read (1937) ascribed some usefulness to the cholera-red test, if used in combination with other biochemical methods.

**Saccharolytic effects**

While, as shown by the tabulation below, *V. cholerae* has been found capable of causing acidification of media into which certain carbohydrates had been incorporated, it has to be emphasized that this process is never accompanied by the formation of gas.

Though the whole of the available literature has been considered for compilation of the tabulation, it is based mainly upon data furnished by Heiberg (1934), because this worker used a satisfactory modern technique: (a) growing the strains to be tested in peptone water (pH 8.0–8.4) into which the various carbohydrates had been incorporated at a concentration of 0.5%; (b) adding bromothymol as indicator in place of litmus (which—as first shown by Müller (1899)—is apt eventually to become reduced by *V. cholerae*); and (c) taking initial readings not later than after an incubation of 20 hours at 37°C so as to be able to distinguish between rapid and late acidification.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Reaction Producing Acidification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arbutin</td>
<td>Constant and rapid acidification</td>
</tr>
<tr>
<td>Dextrin</td>
<td>Late acidification</td>
</tr>
<tr>
<td>Erythritol</td>
<td>No change</td>
</tr>
<tr>
<td>Galactose</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>Glycogen</td>
<td></td>
</tr>
<tr>
<td>Leucrose</td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td></td>
</tr>
<tr>
<td>Saccharose</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td></td>
</tr>
</tbody>
</table>

*a* Late acidification according to Heiberg (1934).

*b* Negative in eight out of nine strains according to Noury & Alalou (1923), variable according to Seal (1935).

*c* See text.

*d* Variable according to some workers.

*e* Variable according to Seal (1935).

As stated by Kauffmann (1934) when reporting upon the observations of Heiberg (1934) referred to later, the reactions produced by individual
cholera strains in carbohydrate-containing media are stable, as proved by re-examination of cultures which had been kept in the laboratory for periods varying from six months to one year. Identical findings have also been recorded by some other workers but the following statements must be noted:

(a) According to observations by Mesnard & Genevray (1931), cholera vibrios which grew on account of variation in the form of opaque colonies with a wrinkled surface produced more vigorous acidification of glucose and saccharose than the parent strains.

(b) Seal (1935) maintained that in general the saccharolytic effects of cholera strains could undergo changes in the course of subcultivation, probably hand in hand with variation of the organisms themselves, a disso­cient of a typical smooth strain in particular producing some acidification only in the presence of glucose.

An elaborate attempt to use tests with carbohydrate-containing media for the classification of cholera and cholera-like vibrios was made by Heiberg (1934), who established that it was sufficient to use three substances only, namely, saccharose, arabinose, and mannose, for this purpose. The results obtained in this manner by Heiberg were thus summarized by Kauffmann (1934) in the *Bulletin de l'Office international d'Hygiène publique*:

<table>
<thead>
<tr>
<th>Group</th>
<th>Saccharose</th>
<th>Arabinose</th>
<th>Mannose</th>
<th>Strains agglutinating with cholera-immune serum</th>
<th>Strains not agglutinating with specific serum</th>
<th>Spontaneously agglutinating strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>239</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>76</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>VI</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

It will be noted that, with the doubtful exception of one atypical and weakly agglutinating strain, the true cholera vibrios fell in Heiberg's Group I. The vibrios not agglutinating with cholera-immune serum, on the contrary, fell in all six groups, far more in Groups II and III than in Group I, which seemed thus a class rather characteristic of *V. cholerae*.

Workers in the cholera areas of India and China soon confirmed that practically always the true cholera vibrios belonged to Heiberg's Group I. In fact, the only observers recording some aberrant results were Seal (1935) in India, and Yu (1938) in China.

Seal maintained that (a) some cholera strains isolated from carriers failed to produce acidity in saccharose-containing media, and (b) arabinose was affected by a very small percentage of cholera vibrios.

Yu in 1938, examining 52 smooth cholera strains which had been isolated during the Shanghai epidemics of 1932 and 1937, found evidence of late arabinose fermentation in some instances, and noted that three of the 1937 strains failed to acidify mannose and
—if the present author may venture to correct a probable misprint—apparently also saccharose even after incubation for seven days.

It has to be emphasized, however, that numerous other workers, when examining freshly isolated strains, never met such aberrant reactions. At the same time it was established, however, that a considerable number of cholera-like vibrios, including those isolated from surface waters, also gave the Group I reactions. Thus Pollitzer (1936) found that practically one third (32) of 100 Shanghai water vibrios belonged to this group. Taylor, Read & Pandit (1936), comparing the reactions of 125 cholera strains with those of 369 cholera-like strains isolated from patients showing clinical signs of the disease, from carriers, and from surface waters, found that 25.8% of the cholera-like vibrios from human sources as well as 11.4% of the water vibrios showed the reactions of Heiberg’s Group I and concluded “that fermentation tests with these three sugars will not provide accurate information as to the characteristics of the vibrios which can be obtained by serological tests”. However, as noted below, in a subsequent paper (1937), Taylor and his co-workers ascribed some usefulness to Heiberg’s method if used in combination with other biochemical tests. Possibly also, as claimed by Heiberg, the method will prove of value for the classification of the cholera-like vibrios which are rather heterogeneous serologically.

Voges-Proskauer reaction

In the course of a study on the bacteria of the haemorrhagic septicaemia group, Voges & Proskauer (1898) found that a red colour was produced if a few drops of a strong solution of potassium hydrate were added to growths of these organisms in glucose-containing media. As was afterwards established, the reaction depends upon the formation of acetylcarbinol in the course of glucose decomposition, which has been ascribed to the action of a special enzyme, “carboligase” (see O’Meara, 1931).

Lemoigne (1920), using a rather delicate reaction (nickel-dimethylglyoxine test) for the examination of culture distillates, found that small quantities of acetylcarbinol were formed by both cholera and cholera-like vibrios. However, while his method thus appeared to have no differential diagnostic value, it was in Lemoigne’s opinion potentially useful for the characterization of different races of these organisms.

Attention to the possibility of using the Voges-Proskauer reaction for the examination of cholera and cholera-like vibrios was, as far as could be established, first drawn by Taylor in a report to the Scientific Advisory Board of the Indian Research Fund Association rendered in 1936 and quoted by Baars (1938). Reporting in detail on these investigations in a valuable Study of the vibrio group and its relation to cholera, Taylor, Pandit & Read (1937) stated that they had compared the modified Voges-Proskauer
reaction according to Barritt (1936) with the original method, using the following technique:

(a) For Barritt's modified test the glucose phosphate medium recommended by the Ministry of Health (Report No. 71, 1934) with an initial pH of 7.5 was distributed in 6" × 5/8" tubes. These were inoculated rather heavily and incubated at 37°C for 3 days. About one ml of the culture was then transferred to a tube and to it was added, first, 0.6 ml of a 5% alcoholic solution of α-naphthol and then 0.2 ml of a 40% KOH solution. Results were read at the end of 4 hours. A positive result was indicated by the appearance of a pink colour on the surface of the fluid in about 5-10 minutes already, which then deepened and spread to the bottom of the tube. In negative cases the fluid usually remained colourless but sometimes a faint brownish tinge appeared.

(b) To carry out the original test, 40% KOH solution was added to the culture tubes in amounts of 0.25 ml after the transfers necessary for the α-naphthol tests had been made. Results were read after 4 and once more after 24 hours.

Carrying out these tests with 90 classical cholera strains, 6 El Tor strains and 351 cholera-like strains, Taylor, Pandit & Read obtained in many instances a positive result with the aid of Barritt’s modified technique only. The reverse, i.e., a positive result with the original Voges-Proskauer technique and a negative one with Barritt’s modification, was never observed.

Combining the observations they made with the aid of Barritt’s test, the cholera-red reaction and sugar fermentation tests according to Heiberg, Taylor and colleagues obtained the following important results:

(1) Classical V. cholerae. All non-haemolytic strains agglutinable with cholera immune serum, belonging to Heiberg’s Group I, gave a cholera-red reaction, but were negative to the modified Voges-Proskauer test.

(2) El Tor strains. Out of the six haemolytic strains which were agglutinable with cholera immune serum, five differed from the classical type in so far as they gave a positive modified Voges-Proskauer reaction.

(3) Cholera-like vibrios. The vibrios inagglutinable with cholera immune serum were found to fall into two main groups:

(a) A larger group (240 strains), showing both a positive cholera-red and modified Voges-Proskauer reaction and consisting mainly of strains of Heiberg Groups I and II;

(b) A minority, cholera-red and Voges-Proskauer negative, belonging with few exceptions to Heiberg Groups III-VI, while one strain was found to fall into a hitherto unknown fermentation group (saccharose negative, arabinose, and mannose positive).

Commenting on these observations, Taylor, Pandit & Read stated the following:

"Mention has already been made that agglutinable non-haemolytic vibrios tested gave the reaction C-R (cholera-red) + V-P (Voges-Proskauer). In the series of 351 inagglutinable strains examined, only 15 gave the same results and of these 10 were of types aberrant in their sugar reactions from the recognized Heiberg types. No inagglutinable strain of
Heiberg type I has given the reactions C-R + V-P-. It is therefore possible, on biochemical evidence alone, to obtain presumptive diagnosis of the serology of the typical *V. cholerae*; if it gives fermentation reactions of Heiberg type I, is cholera-red positive and negative to the modified V-P test, it is very probably an agglutinable vibrio."

Taylor, Pandit & Read claimed in this connexion that, if rather large inocula were used, it was permissible to carry out Barritt's test after an incubation of only one day instead of the customary three days and that consequently the fermentation, cholera-red and modified Voges-Proskauer tests could be "profitably performed, along with the agglutination test and read with it". It has to be pointed out, however, that, using the now available sera, great reliance can be placed upon slide-agglutination tests made as soon as suspicious colonies are found on the plates used for primary isolation of *V. cholerae*.

The important observation that, in contrast to most classical *V. cholerae* strains, the El Tor vibrios usually give a positive Voges-Proskauer reaction, was confirmed by several workers, such as de Moor (1938, 1949), Mochtar & Baars (1938), Gispen (1939), Marras (1940) and Paris & Gallut (1951). Baars (1940) maintained in this connexion that the El Tor vibrios were capable of forming acetylmethylcarbinol only under aerobic but not under anaerobic conditions. Gallut (1946) found, like Lemoigne in 1920, that, if tests more sensitive than the Voges-Proskauer reaction were used, the cholera vibrios could be also proved to produce this substance. However, the El Tor vibrios acted far more energetically in this respect. This is in accord with the observation of Baars (1940) that these vibrios ferment sugars far more energetically than the *V. cholerae* under aerobic conditions and to some extent even under anaerobic conditions.

**Haemodigestive and haemolytic properties**

Observations on the reactions produced by *V. cholerae* in blood-containing media go back to a rather casual statement made in 1884 by Koch at a cholera conference in Berlin (*Berliner klinische Wochenschrift*, 1884) to the effect that in one instance, when blood-containing stools had been used to make a gelatin plate, clear zones became visible round the cholera colonies. Koch felt entitled to conclude from this observation that *V. cholerae* was capable of destroying erythrocytes and probably also other cells. Schottmüller (1904) also ascribed haemolytic properties to the cholera vibrios which facilitated the differentiation of these organisms from other intestinal bacteria.

The observations of Bitter (1886) on the action of the "ferment" of *V. cholerae* on rabbit blood-suspensions cannot be considered conclusive because he worked with culture fluids which had been heated for half an hour at 60°C. He found that under these circumstances the erythrocytes were remarkably resistant to the action of the ferment. The haemolytic action of *V. cholerae* on blood-containing gelatin plates, ascribed by other
workers to the secretion of a cell-destroying toxin by the organisms, was in Bitter’s opinion due to the damage caused to the erythrocytes through enclosure in the media, on account of which the blood corpuscles became amenable to the action of the ferment and other products of decomposition.

An unequivocal claim that *V. cholerae*, like some other micro-organisms, produced a haemolytic enzyme was made by Eijkman (1901), but there can be no doubt that the halo formation on blood-agar plates described by him was the result of haemodigestion (see below) and not of true haemolysis.

Studying 12 cholera-like as well as 9 true cholera strains, Kraus (1903) found the former alone capable of producing a soluble “haemotoxin” in broth cultures and consequently able to produce zones of clearing round their colonies on blood-agar plates. Kraus recommended, therefore, the use of the latter media for the differentiation of the non-haemolytic *V. cholerae* from haemolytic cholera-like organisms.

The problem of the haemolytic properties of the vibrios began to attract much attention after Gotschlich (1905, 1906) had isolated six peculiar strains from dead bodies of returned Mecca pilgrims at the quarantine camp of El Tor. Though these victims showed no signs of choleraic disease either during life or post mortem, the vibrios found in their intestines were not only agglutinable with cholera-immune serum but showed, as far as the tests used by Gotschlich went, in all other respects as well, the reactions of true cholera vibrios. However, re-examining these strains, Kraus & Pribram (1905) found to their surprise that the organisms in question produced, like the cholera-like vibrios formerly examined by Kraus, a soluble haemotoxin as well as an exotoxin rapidly lethal to experimental animals.

Since this discovery was made, diametrically opposite views have been expressed in regard to the relationship between these El Tor vibrios with the true cholera vibrios responsible for epidemics, and—in connexion with this problem—regarding the question whether or not the classical *V. cholerae* is non-haemolytic in contrast to the El Tor vibrios. Kraus and his co-workers (see the ultimate statement of Kraus, 1922) continued to assert that on account of its above-described properties the El Tor vibrios fell into a class distinct from that of the non-haemolytic *V. cholerae*. Many German workers on the contrary (see summary by Kolle & Prigge, 1928), maintained that the cholera vibrios were apt to show variability in regard to their haemolytic properties as well as in other respects and that, consequently, tests with blood-containing media were unsuitable for the characterization of this organism—an opinion which implies that the El Tor vibrios do not form a group of their own.

1 Though a few workers have designated also haemolytic cholera-like vibrios with this name, it is imperative to use it exclusively for those haemolytic strains which are agglutinable with cholera-immune serum. Otherwise utter confusion would reign.
In order properly to assess the merits of these opposite claims, it is necessary to pay attention to the methods of examination used by the various workers and to the manner in which they interpreted their findings.

Proper choice of blood

The first point to be noted in this connexion is that the various workers have used different sorts of erythrocytes for their tests. As noted above, Koch (1884) made his initial observation on the supposed haemolytic properties of *V. cholerae* on a plate which happened to contain human blood. The use of this was recommended by Schottmüller (1904), while some other early workers (e.g., initially Kraus, 1903) used rabbit blood for their tests. Prausnitz (1905), who seems to have been the first to make comparative tests in this respect, found rabbit as well as calf blood more suitable than human blood, but worked for the sake of economy mainly with calf blood. The use of the latter was strongly recommended by Schumacher (1906), because in his experience the calf erythrocytes were the least liable to become damaged by mechanical, thermic, or chemical influences and were, therefore, the most resistant to the action of the vibrio "ferments". Kraus and his co-workers on the other hand (see Kraus & Prantschoff, 1906) soon adopted the use of sheep blood but considered goat blood also suitable. Goat blood has been used for the large-scale studies on the haemolytic properties of *V. cholerae* referred to below, but, as confirmed by some later observers, for instance on account of comparative tests by Finkelstein (1930), sheep blood was equally satisfactory. In fact, Krishnan & Gupta (1949), submitting in 1949 to the WHO Expert Committee on Cholera a draft proposal for a standard haemolytic test to be adopted for cholera work, recommended the use of sheep blood in preference to that of goat blood.

These statements make it clear that in assessing the results of past workers, full reliance can be placed only on findings with suitable types of blood, particularly with goat, sheep, or calf blood, while those with human blood ought to be disregarded. It is of great interest to add that according to observations made by Zimmermann (1934) most cholera strains, though incapable of producing lysis of sheep erythrocytes, were found able to form a thermolabile haemolysin against human red blood-corpuscles, while the El Tor vibrios lysed both sorts of blood. These observations, which have been recently confirmed by De and co-authors (1954), are in accordance with earlier findings made by Pribram & Russ (quoted by Kolle & Schürmann, 1912 and Kolle & Prigge, 1928) who, carrying out absorption tests, showed that the filtrates of vibrio cultures did not contain one common haemolysin but separate ones for the different sorts of erythrocytes they were able to lyse.
Methods of examination

Two fundamentally different methods are used to assess the behaviour of cholera or other vibrios in blood-containing media—cultivation of the organisms on blood plates (nowadays invariably agar plates) and tests with blood suspensions which have been added to adequate amounts of fluid vibrio cultures, or of their filtrates or centrifugates. The technique usually adopted for the latter purpose which, as will be set forth in a later chapter, is still used in actual practice with some modification, is well exemplified by the following description of the classical procedure adopted by Greig (1914b):

"Each strain was grown in alkaline broth, as recommended by Meinicke (1905) for 3 days at 37°C., at the end of that period falling quantities of the culture, viz., 1 c.c., 0.5 c.c., 0.1 c.c., 0.05 c.c. and 0.01 c.c. were measured with a pipette and placed in small sterile test-tubes; the quantities were brought up to exactly 1 c.c. in each tube with 0.85% NaCl. Then 1 c.c. of a 5% suspension of goat’s washed red corpuscles was added to each tube. An experimental error is made if the suspension of red corpuscles is added first, since the culture, which is lighter, floats on the top; so that if a haemotoxine is present the upper layer of red corpuscles gets a very concentrated dose. The contents of the tube are very carefully mixed and the mixture is placed in the incubator at 37°C. for 2 hours. The tubes are taken out and placed in the ice-chest over night. Next day the presence or absence of haemolysis in each tube is noted and recorded."

No doubt, it would be more exact to use corresponding amounts of filtrates instead of materials from the fluid cultures for the above-described tests. Unfortunately, however, as first shown by Meinicke (1905) and confirmed by later observers, the haemolytic property of the strains is greatly reduced if filtration is resorted to. Greig (1914b) maintained in this respect that “the haemolysis-producing substance in the broth culture is, to a considerable extent, non-filterable”.

It will be perceived that, whereas in the case of tests performed according to Greig’s or a similar technique the red blood corpuscles are exposed almost solely to the action of the “haemotoxins” (haemolysins), in the case of cultivation on solid blood-containing media, they are also exposed to the action of the enzymes of the vibrios. It is not surprising, therefore, that, as will be shown below, the results obtained with these two categories of tests respectively are as markedly different as the technique adopted in each case. It is obvious that the results of tests aiming to show the presence or absence of haemolysis will be far more clear-cut if, by using Greig’s or a similar technique, or by working with filtrates, the additional influence of the vibrio enzymes is practically or totally excluded.

Quality of the media used

As noted by Schumacher (1906) in the course of exhaustive studies on the behaviour of cholera and cholera-like vibrios on blood-agar plates, it is essential to pour these with a sufficient amount of the medium so as
to obtain a uniformly and adequately thick layer. The reason for this was that even vibrios, which ordinarily did not produce zones on the plates, were apt to show ill-defined haloes round their colonies at thin spots of improperly poured plates. Loewy (1915), besides repeating the advice given by Schumacher, also insisted upon the use of freshly taken and defibrinated blood, because blood kept in storage could show spontaneous haemolysis. Plates which had become dry or which showed a darkening of their initially bright-red colour were unsuitable for haemolysis tests.

Zimmermann (1932) noted, in analogy with the experiences of Meinicke (1905) in the case of blood plates, that the results of haemolysin tests, made by growing cholera vibrios for 48 hours in broth tubes to which sheep blood had been added previously to obtain a concentration of 5%, were apt to be divergent if the tests were repeated at short intervals. It was striking, however, that different strains tested at one and the same time showed a peculiarly uniform behaviour, either mostly producing haemolysis or mostly failing to do so. Since such a simultaneously occurring variation of several strains was altogether unlikely, Zimmermann postulated with much reason that the observed variations in the haemolytic properties of the strains were the result of differences in the physico-chemical state of the media or of corresponding changes taking place in the course of cultivation. Inadequacies in the defibrination of the blood were likewise apt to introduce an element of chance. In fact, consistently negative results were obtained with the same strains if, instead of the broth, a synthetic fluid medium and, instead of defibrinated blood, citrated sterile sheep blood were used. However, significant though these findings are, in actual practice it is equally reliable and more expedient to use an up-to-date modification of Greig's method in place of that of Zimmermann. It seems unnecessary, therefore, to deal in detail with the technique of the last-mentioned worker.

Status of the strains examined

The various workers postulating an inconstancy of the reactions produced by vibrios in blood-containing media or suspensions based their claims, to a varying extent, upon an examination of recently isolated growths and of stock cultures respectively. It is of importance, therefore, to see whether, or to what extent, the inconstancies which they noted in the course of their work were due to a changing reactivity of the individual strains, caused by the process of ageing and/or by mutation or dissociation.

Studying the mutations of V. cholerae, Baerthlein (1911b, 1912, 1918) noted that the opaque variants of ordinarily non-haemolytic cholera vibrios were able to produce haemolysis in blood suspensions as well as clear zones round their colonies on blood-agar plates. Since, however, this worker continued to keep both the suspensions and the plates at 37°C
in the incubator and extended the period of observation to 72 hours, no reliance can be placed on his findings. Further observations made by Goyle & Gupta (1932) with spontaneously agglutinating cholera strains which had obviously undergone dissociation, and by Genevray (1940) with variants of \textit{V. cholerae} obtained through the action of chlorine or phenol showed that, like their smooth parent-strains, these dissociants and variants respectively failed to produce haemolysis in blood suspensions.

Van Loghem (1913b) and Snapper (1921) asserted in general that, while the haemodigestive properties of \textit{V. cholerae} were apt to show variation, the incapability of these organisms of producing haemolysis in fluid substrates, as well as the haemolytic properties of the El Tor vibrios, were stable characteristics. This is in accord with previous observations made by Meinicke (1905) who stated that

"the haemolysins of the vibrios are but little apt to undergo spontaneous decomposition. Out of 20 filtrates of different vibrios cultures, to which phenol had been added and which had then been kept in the ice-box for 6 months, 17 retained their original titre and three only had become less haemolytic." [Trans.]

Analogous observations were made by Zimmermann (1933), who found that, with the exception of one variable El Tor strain, none of the vibrio strains studied by him showed evidence of a short-term variation of their haemolytic properties. Re-examining these strains once more after a period of observation totalling one year and nine months, Zimmermann (1934) likewise observed no instance of a fundamental change in their haemolytic properties, and only in a limited number of instances a variation in the intensity of the reactions produced by haemolytic strains. The haemolytic properties of Zimmermann's strains were not influenced by animal passage, nor by bacteriophage action as had been claimed by Doorenbos (1932).

Though, as will be gathered from the evidence set forth above, the presence or absence of haemolysis may be considered stable characteristics of practically all strains of the cholera and allied vibrios, it is nevertheless desirable to use freshly isolated growths rather than stock cultures to assess the reactions produced in this respect by a given strain or series of strains. Van Loghem (quoted by Zimmermann, 1932) no doubt went rather far when ascribing the occurrence of aberrant haemolytic reactions shown by stock cultures of \textit{V. cholerae} to contaminations with haemolytic vibrios. But even apart from this possibility, the uncertainties arising from the use of stock cultures the source of origin and character of which are quite often not or not exactly known may be great, and this absence of exact information was no doubt responsible to quite a considerable extent for the statements made to the effect that the classical \textit{V. cholerae} may produce true haemolysis. The difficulties apt to arise in

\footnote{The only recent observations recorded to the contrary were those of Del Favero (1938) who stated that laboratory strains of \textit{V. cholerae}, subjected 15 times to subcultivation at 20°C, became, in contrast to their initial behaviour, strongly haemolytic for sheep-erythrocytes. Since Del Favero's original paper could not be consulted, details of his methods could not be ascertained.}
this respect are well exemplified by the experiences of Zimmermann (1932). This worker found among the 70 strains labelled in his material as *V. cholerae* two which were not agglutinable with cholera-immune serum but were haemolytic, and which, therefore, as he cautiously put it, could not be considered "typical" cholera vibrios. Out of Zimmermann's 21 strains labelled as El Tor, on the other hand, one proved to be non-haemolytic, thus reacting like a cholera vibrio and not like an El Tor vibrio. A minority of his other El Tor strains were but slightly or even almost not agglutinable with cholera-immune serum, but—as Zimmermann argued—"they must have been found agglutinable with cholera serum by Gotschlich and Doorenbos, because they had been diagnosed on account of this fact".

**Interpretation of results**

The fundamental difference between the phenomenon of true haemolysis observable in fluid substrates and the appearances apt to become manifest when the vibrios were grown on blood-plates was clearly recognized by Schumacher (1906), who maintained in this respect that:

"there can be no doubt that the halo formation of cholera strains on blood agar prepared with human, pigeon, rabbit, guinea-pig, horse, and dog blood is not due to a haemolysin production by the cholera colonies, but is solely due to the action of the proteolytic ferment excreted by the cholera bacteria". [Trans.]

However, even though Schumacher and also some other early workers emphasized that, in order to decide whether or not a given strain was haemolytic, tests should be made with blood suspensions and not with blood plates, many investigators not only mainly paid attention to the latter category of tests but often took the appearance of clear zones round the vibrio colonies on the blood plates as proof that the strains in question were endowed with haemolytic properties.

It was the great merit of Van Loghem (1911, 1913a, 1913b) to have reaffirmed through studies commenced in about 1909 that the classical cholera and the El Tor vibrios respectively produced qualitatively distinct reactions in blood-agar plates besides being distinguishable by their behaviour in blood-containing fluid substrates, in which, in contrast to *V. cholerae*, the El Tors produced haemolysis (Fig. 19). It is true that *V. cholerae* was capable of producing clear zones round its colonies on goat-blood agar plates as the El Tor vibrios invariably did. However, Van Loghem emphasized, in the case of the latter organisms, that these zones appeared quickly, were not quite transparent, and showed a reddish tint. In the case of *V. cholerae*, on the contrary, the zones appeared more slowly, were quite clear and had a greenish hue. Spectroscopically, it could be shown that oxyhaemoglobin, while absent in the zones around the cholera colonies, was present in the zones surrounding the El Tor colonies, because in their case true haemolysis took place which led to the penetration of haemoglobin into the zones. The
FIG. 20. VIBRIO CHOLERAE GROWN ON BEEF INFUSION AGAR SLANT FOR 18 HOURS AT 37°C, BEFORE (A) AND AFTER (B) SUPERSONIC TREATMENT (WHOLE-CELL VACCINE)

Palladium-shadowed; x 8000

A

B
FIG. 19. HAEMOLYSIS AND HAEMODIGESTION OF 48-HOUR-OLD VIBRIO CULTURES IN PETRI DISHES ON NUTRIENT AGAR WITH 5% SHEEP RED CELLS

Streak seeding

Left: Haemolytic El Tor vibrio, non-haemodigestive
Right: Haemodigestive Vibrio cholerae, non-haemolytic

Spot seeding

Left: Haemolytic El Tor vibrio, non-haemodigestive
Right: Haemodigestive Vibrio cholerae, non-haemolytic

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process of haemodigestion, of which alone the cholera vibrios were capable, led to a decomposition (Abbau) of the components of the erythrocytes and, as a consequence, no haemoglobin was present in the zones.

Haemolysis, produced by the El Tor vibrios, was an eminently stable property of the organisms; haemodigestion was an unstable property possessed by the *V. cholerae* as well as by the El Tor vibrios. Van Loghem found a close parallelism between the haemodigestive and gelatin-liquefying properties of the various vibrio strains, which indeed, in the opinion of some observers, are due to the action of one and the same enzyme.

The old El Tor strains of Van Loghem's series liquefied gelatin very slowly and it seemed likely, therefore, that in their case the process of haemolysis quite overshadowed that of haemodigestion.\(^1\)

The validity of Van Loghem's findings was questioned by Baerthlein (1914, 1918), but since the conclusions of the latter were disproved by Snapper (1921), it seems unnecessary to discuss Baerthlein's rather involved postulations in detail. Van Loghem's observations were confirmed by Kämmerer in the course of a profound study on bacteria and red blood corpuscles (1920) and supported as well as amplified by Snapper (1918, 1921), who in 1921 thus summarized his interesting findings:

"[a] In the halo round a cholera culture on a blood-agar plate and in the culture itself haematin is formed; consequently the halo is produced through a decomposition of haemoglobin.

"In the halo round an El Tor culture at most traces of haematin are formed; the halo is produced through haemolysis and diffusion of the liberated haemoglobin throughout the plate. As proof for this serves that on haemoglobin plates, which contain only dissolved blood-colouring substances, the El Tor vibrios form no halo, the cholera vibrios a distinct halo.

"[b] In El Tor growths on blood-agar plates inorganic iron compounds are formed, whereas in the cholera growths no inorganic iron but haematin is produced.

"[c] The halo-formation by the cholera bacilli is much more marked on blood-bile-agar than on ordinary blood plates. On the former even cholera strains which seem to have lost the ability of forming haloes, can still produce wide haloes, in which a further decomposition of the haematin spontaneously formed in such plates takes place.

"El Tor vibrios form on blood-bile-agar plates usually no, exceptionally indistinct haloes." [Trans.]

In Snapper's opinion the explanation of the last-mentioned phenomenon was that in the bile-containing blood-agar all haemoglobin had been transformed into haematin substances, no unchanged erythrocytes having been left.

Final confirmation of Van Loghem's findings was furnished through a series of fine studies by Bernard, Guillerm & Gallut in 1937. These workers were able to extract from agar and broth media used for the cultivation of *V. cholerae* a protease which (a) while possessing no haemolytic power was

\(^1\) Further reference to Van Loghem's observations on cholera and El Tor vibrios will be made in the following chapter where the vibrio haemolysins will be dealt with from the immunological point of view.
found to be capable of bringing about in successive stages “the phenomenon of haemodigestion” as described by Van Loghem, and (b) was also found to exert a tryptic action both on denatured proteins, such as coagulated horse-serum, gelatin, and milk, and natural proteins such as egg-white and fibrin. The results of these investigations as well as those of the extraction of an exohaemolysin from culture media used for growing El Tor vibrios, which were recorded by Bernard, Guillerm & Gallut in 1939 and will receive full attention in the next chapter, definitely confirm the difference of the reactions produced by cholera and El Tor vibrios in blood-containing media or substrates, thus ending a controversy lasting for more than thirty years.

The experiences of Greig (1914b) and other modern workers in testing representative series of vibrio strains, almost invariably1 supported the validity of the above-mentioned observations.

Greig (1914b) found that the 333 cholera strains which he examined according to the above-mentioned technique all proved non-haemolytic, while 100 strains of cholera-like vibrios, isolated from human stools or from surface waters, invariably produced haemolysis, some to a marked degree. A great majority of the 161 cholera strains, the behaviour of which was tested on agar plates containing 12% goat-blood, produced no definite zones of clearing in these media within 24 hours, and only a few indistinct ones. However, clear zones became manifest, if readings were taken after more prolonged incubation. Greig emphasized, therefore, that, if blood plates were used to test the haemolytic properties of cholera-suspect vibrios, positive findings becoming manifest after more than 24 hours should be disregarded.

Testing 103 cholera strains which included, besides 27 stock cultures, mainly those isolated in Romania and Bulgaria, Loewy (1915) found no evidence of haemolysin production either in the centrifugate of 5 days’ broth cultures or in the case of agar plates to which 10% sheep or goat blood had been added. However, a proteolytic “ferment” of the V. cholerae, which in Loewy’s opinion was identical with the ferment causing gelatin liquefaction, was found by this worker to be capable of exerting a digestive action on damaged erythrocytes.

Van Loghem, summarizing in 1932 the results of the above-mentioned observers as well as those obtained by various workers in the Dutch East Indies, was able to report on the examination of over 600 strains isolated from authentic cholera cases and invariably found to be non-haemolytic.

As Zimmermann summarized in 1933, among the 69 cholera strains examined by him, two were found to possess haemolytic properties. With one exception, which has been noted above, the 28 El Tor strains of his col-

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1 A divergent opinion was expressed by Kabeshima who, in a short note published in 1918, maintained that 91.6% of the 206 cholera strains investigated by him showed haemolysis in fluid as well as on solid media. It was not possible to consult the more exhaustive publication in a Japanese medical journal which Kabeshima referred to in his note. There can be hardly any doubt, however, that deviations from the standard technique used by the other workers were responsible for the strikingly aberrant results obtained by him.
lection produced marked haemolysis and the same was true of the 14 cholera-like strains at his disposal. Of the two haemolytic cholera strains, one was a stock strain over 20 years old which agglutinated but weakly with cholera-immune serum, the other a stock strain from Paris kept in the Berlin Institute of Hygiene.

Genevray & Bruneau (1938c), recording their observations on more than 500 Indochinese cholera strains, stated that they had observed no instance of haemolysis produced by these vibrios within 24 hours. However, all the strains produced hémolyse (that is, apparently, haemodigestion) on sheep blood as well as on rabbit blood agar.

Attention has also to be drawn to the experiments made by a few workers with heated blood-agar plates ("chocolate" agar plates), on which, as was first noted by Loewy (1915), cholera vibrios were apt to produce clear zones. Kovacs (1927), exhaustively studying this phenomenon, confirmed that these vibrios, obviously because they were capable of exerting an action on the erythrocytes which were damaged through boiling, produced—usually on the second day of incubation—yellowish, quite transparent haloes round their colonies on chocolate agar (Kochblutagar) plates prepared with the aid of sheep blood. El Tor vibrios on the contrary, because they exerted at most a slight haemodigestive action, produced but occasionally indistinct haloes.

Finkelstein (1930) postulated that by the combined use of blood suspensions, ordinary and heated blood-agar the vibrios could be classified into four groups thus:

<table>
<thead>
<tr>
<th>Group</th>
<th>Haemolysis in suspensions</th>
<th>Clearing of blood-agar</th>
<th>Clearing of heated blood-agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>II</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>III</td>
<td>positive</td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>IV</td>
<td>negative</td>
<td>positive</td>
<td>positive</td>
</tr>
</tbody>
</table>

In contrast with the findings of Loewy and Kovacs, Finkelstein claimed that the classical *V. cholerae* does not produce clearing of heated blood-agar plates, thus falling into Group I of his scheme. Since, however, he used ox blood for his tests, and took readings after 24 hours' incubation at 37°C only, and since, moreover, a study of his protocols shows that out of the total of 11 strains isolated from cases of clinical cholera which he could examine, 5 only fell into Group I, while 3 belonged to Group II, 2 to Group III and 1 to Group IV, it is impossible to place reliance in Finkelstein's findings. Generally speaking, it must be emphasized once more that tests on blood plates, however interesting their results may be, are of no decisive value in answering the practically most important question of whether or not a given vibrio strain is capable of producing true haemolysis. Tests with blood suspensions alone can furnish clear-cut evidence in this respect.
In addition to the above-recorded findings, within recent years the following important observations have been made in regard to the El Tor vibrio in particular.

As has been noted in Chapter 2, in 1937-38 as well as in 1939-40 and in 1944 manifestations of a "choleriform" disease with a high fatality rate have been observed in South Celebes, in which haemolytic vibrios agglutinable with cholera-immune serum were found to play the causative role (de Moor, 1938, 1949). Making a careful study of 370 strains isolated from sufferers and their environment, de Moor (1949) concluded that the vibrios in question, which fell in the same serological group as *V. cholerae* and belonged like the latter to Group I of Heiberg, but gave with four exceptions a positive Voges-Proskauer reaction, were true El Tor vibrios. Agreeing with Van Loghem (1938) that the El Tor vibrio fell into a group different from that of *V. cholerae*, de Moor proposed the name "Paracholera (El Tor)" for the choleraic disease in Celebes. It is thus curious to see this term once more used in the sense proposed by Kraus (1909), i.e., to designate instances of choleraic disease caused by haemolytic vibrios agglutinable with cholera-immune serum. Since, however, the name "paracholera" was afterwards adopted to designate clinical manifestations in which vibrios serologically different from *V. cholerae* were assumed to have played a causative role, it cannot be considered as felicitous. The term "enteritis choleriformis El Tor" proposed by Van Loghem (1938) to designate the Celebes disease seems therefore preferable.

Though the outbreaks observed in Celebes have been the most conspicuous, they were probably not the first in which El Tor vibrios were responsible for the causation of choleraic disease. A strain called Kadikój, which showed the properties characteristic of the El Tor vibrios, was isolated in 1913 by Kraus in Bulgaria (Kovacs, 1927), while Hoppe-Seyler (1916) claimed that cholera vibrios with haemolytic properties had been responsible for a limited outbreak in Kiel and added that *V. cholerae* strains of this kind had been met with repeatedly in Poland during the First World War. Mackie (1929) maintained in general that vibrios of this type had been met in choleraic conditions as well as in carriers in countries outside India, e.g., in the Near East.

It is of great interest to note in the latter connexion that Abdoelrachman (1944-45), examining 90 water samples from different sources in the Hejaz, was able to demonstrate the presence of El Tor vibrios in one out of 29 specimens taken from the holy well Zam-Zam at Mecca. All other water samples examined by this worker as well as 1109 stool samples, including those of 715 pilgrims from the Dutch East Indies and Malaya, gave negative results for the El Tor vibrio and, cholera being absent at the time, also for *V. cholerae*.

1 See page 64.
Recent noteworthy observations on the occurrence of El Tor vibrios in India may be summarized as follows. Venkatraman and co-authors (1941) recorded that they isolated 15 El Tor strains from 878 specimens, collected in the Tanjore district of Madras from 237 open natural water-sources, including rivers, channels, tanks, ponds and a few wells. The examination of 1827 stool samples gave negative results for El Tor vibrios and, since cholera was absent at the time, also for V. cholerae.

Read & Pandit (1941) carried out analogous investigations in (a) two districts of Bengal, where cholera was endemic; (b) a district in Bihar, where annual epidemics occurred; and (c) an area in Sind, which had remained largely free from cholera for the past ten years. The main conclusions reached by these two workers were:

"[1] The non-haemolytic agglutinable vibrio was found in all except one of the clinical cases in areas where the presence of cholera could be established, provided the examination was carried out sufficiently early in the disease.

"[2] About 7 per cent of close contacts of cholera cases proved positive and about 16 per cent of water sources in direct contact with cases were positive at different periods of the epidemic. On the other hand the non-haemolytic vibrio with one or possibly two exceptions was not found (in water samples) in the absence of the disease.

"[3] The haemolytic agglutinable vibrio, while detected in the presence of the disease, has been found usually in its absence. It has been found in cholera areas of two different epidemiological types in different provinces of India and in relative large numbers (i.e. in 18 out of 206 water samples) in an area which must be taken as not only free from cholera during the period of investigation but free from cholera during the decade previous."

Continuing these investigations, Read, Pandit & Das (1942) tested the haemolytic properties of cholera and El Tor strains from various sources. Growing these organisms in Douglas's broth (see Douglas, 1914) instead of in untrypsinized broth as had been done by Greig, but otherwise using the technique recommended by the latter worker, Read, Pandit & Das obtained the following results:

<table>
<thead>
<tr>
<th>Character of strains</th>
<th>Haemolysis produced</th>
<th>Haemolysis not produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case strains, India</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Case strains, Celebes</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Contacts, India</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Contacts, Celebes</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Water, India</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>Water, Celebes</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>El Tor stock strains</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

Total . . . . . 36 26

* All agglutinable with cholera-immune serum.

It will be noted that while the strains isolated from cholera patients and contacts in India, in contrast to those from Celebes, were invariably non-haemolytic, the majority of the strains isolated from water sources in India
showed the haemolytic properties characteristic of the El Tor vibrios. This was particularly true of the growths obtained from "non-contact" water sources, 14 out of 15 of which proved haemolytic.

In marked contrast to these observations, Mukherji (1955) claimed that El-Tor-type vibrios had been responsible for a cholera outbreak at Lucknow in 1945. However, since (a) bacteriological examinations seem to have been made in but one-half of the patients showing clinical signs of the infection, and (b) only 13 of the 25 strains found to be agglutinated by cholera-immune serum proved positive in Greig tests, Mukherji's claim does not seem convincing. It is quite possible that, just as cholera-like vibrios of obviously aquatic origin are quite frequently found side by side with or in place of V. cholerae in the stools of patients showing typical signs of cholera during epidemics, so the presence of the haemolytic vibrios noted by Mukherji was of an accidental nature, being due to the occurrence of this type of organism in the environment of the sufferers. Nevertheless, in view of the observations made in Celebes, it would be unwise to deny the possibility that El-Tor-type vibrios can be responsible for occasional choleraic manifestations in man. At the same time, however, it would seem unjustified thus far to doubt that, as Gardner & Venkatraman put it,

"the absence of haemolytic power and the possession of a characteristic O antigen are the chief distinctive characters of the vibrios most undoubtedly causative in epidemic cholera."

Vital Resistance

Heat

It is generally agreed that V. cholerae is not at all resistant to high temperatures. As Kolle & Schürmann (1912) summarized in this connexion,

"Boiling temperature destroys the vibrios immediately. At 80°C they are killed with certainty within five minutes, and heating for half an hour at 56°C suffices to terminate the life of the cholera vibrios." [Trans.]

Babes (1885) established that rapid heating of gelatin cultures to even only 75°C rendered the growths sterile. Good growths could be obtained from gelatin cultures slowly heated (? in the water-bath) up to 45°C. Exposure at 46°-48°C for two days rendered the cultures sterile, but temperatures of 40°-41°C were well tolerated by the cholera cultures for three days.

Kitasato (1889a), heating gelatin tubes, which after liquefaction had been inoculated with V. cholerae, at various temperatures and for different lengths of time in the water-bath and then making roll cultures, found that (a) exposure of the inoculated tubes for 15 minutes to 55°C usually prevented growth, and (b) heating for 10 minutes at 60°C or for 5 minutes at 65°C invariably did so.
In the experience of Borntraeger (1892) dry heat of 80°C killed the cholera vibrios within a few minutes while exposure to higher degrees of dry heat (80°-100°C) led to the death of the organisms within a few seconds. Borntraeger considered it feasible under these circumstances to use, in emergencies, dry heat generated in baking stoves for the disinfection of objects such as clothing and bedding contaminated with *V. cholerae*.

It is of interest to add that Shousha (1924) found the rough dissociants of *V. cholerae* somewhat more resistant to heat than the smooth parent strains.

**Cold**

Though not very resistant to heat, the cholera vibrios show a remarkable tolerance for low temperatures, even for those well below the freezing-point. Uffelmann (1893a) established in this connexion that suspensions of *V. cholerae* in water as well as cholera cultures, if exposed in the open during winter, remained viable for 3-4 days even when the minimal temperature became as low as −24.8°C. Still more remarkable experiments carried out by Kasansky (1895) showed that broth, gelatin, and agar cultures of *V. cholerae* (a) tolerated temperatures down to −31.8°C, (b) remained viable if kept completely frozen for 20 days or if subjected to repeated freezing and thawing, and (c) survived exposure to the cold of the winter at Kasan, Russia, for four months. However, cultures exposed to the cold in November proved to be no longer viable when re-examined in April or May. The survival of cholera vibrios in artificially contaminated raw river water samples under the conditions prevailing in winter (December to mid May) in Berlin for a maximum period of a little over four months has been recorded by Christian (1908).

**Drying**

As Koch recorded at a cholera conference held in Berlin in 1884 (*Berliner klinische Wochenschrift*, 1884), cholera vibrios, grown in peptone broth and spread in thin layers on cover-glasses, withstood drying for periods of up to one hour, but were sometimes found to have succumbed after two hours and were invariably incapable of surviving drying for periods exceeding three hours. If compact masses of vibrios, scrapings from potato-cultures for instance, were dried, the organisms could survive for periods of up to 24 hours, evidently because under these circumstances no rapid drying took place.

Similar experiments were made by Kitasato (1889a), who found that on silk threads which had been dipped into fluid cultures the cholera...
vibrios withstood drying better than on cover-glasses, obviously because desiccation took place more slowly. Working at room temperature (20°-22°C), Kitasato found that even on the silk threads the vibrios survived for a few days only, longest (up to seven days) on those kept in the exsiccat, apparently because in the latter case rapid drying of the outer layers led to a more prolonged retention of some moisture inside the threads.

However, in an additional note, Kitasato (1890) stated that, if kept on moist filter-paper in closed Petri dishes, cholera vibrios on cover-glasses were capable of surviving for 85-100 days, and for 200 days or even longer on silk threads.

Suzuki (1922), again studying the resistance of V. cholerae to drying, found that if the organisms were smeared on a silk thread which was then placed in a jar with calcium chloride, the organisms survived no longer than 18-28 hours. However, longer survival took place if suspensions of the vibrios in saline solutions containing some horse serum, egg-white, or undiluted horse serum were used for such tests.

Observations made by some workers have shown that, when undergoing exsiccation, cholera vibrios from layers which apparently had become quite dry may remain subcultivable. Thus Gildemeister & Baerthlein (1915), studying the survival of V. cholerae in the faeces of patients and carriers (see below) sometimes obtained positive results when making subcultures from apparently exsiccated specimens.

Laigret & Auburtin (1938) recorded that they had obtained broth subcultures from cholera vibrios which had been dried in vacuo over calcium chloride, ground in a mortar, and then kept in rubber-stoppered test-tubes at temperatures ranging from 25°C to 39°C for five weeks. Still more remarkable results were recorded by Campbell-Renton (1942) who, drying single drops of peptone water cultures of cholera and El Tor vibrios in vacuo over phosphoric oxide (P₂O₅), still obtained positive results when making subcultures from six out of seven V. cholerae and three out of five El Tor specimens tested after four years’ storage in vacuo at room temperature. In contrast to this favourable experience, Shaw (1956), as well as some earlier workers mentioned by her, reported that attempts at preserving cholera cultures with the aid of the spin-freeze method of Greaves (1944) or of similar procedures gave no satisfactory results. However, according to the experiences of several observers (Burrows et al., 1947; Hornibrook, 1949, 1950; Sokhey, 1949; Sokhey & Habbu, 1950) freeze-drying (lyophilization) is an excellent means of preserving not only the viability but also the immunogenic properties of the V. cholerae. While Hornibrook (1950) recommended a menstruum containing only lactose, citrates and inorganic salts as the best for freeze-drying bacteria or viruses, Neogy & Lahiri (1956) stated that skimmed milk was a good suspending medium for the preservation of V. cholerae by the freeze-drying method but that the duration of preservation was never very long.
Sunlight

Orsi (1907), carrying out systematic studies with cultures of *V. cholerae* and *Salmonella typhosa*, found that sunlight exerted a damaging action on these organisms without, however, invariably leading to their total destruction. The cholera vibrios, in particular, remained viable in fairly considerable numbers after exposures to sunlight averaging 8-10 hours (temperature in the shade 23°-31°C). In the experience of Conor (1912), however, these organisms, if suspended in canal water and exposed to the action of sunlight in transparent glass flasks or tubes, were no longer demonstrable after an exposure of 5-7 hours at temperatures ranging from 27°C to 34.6°C. Yasukawa (1933), working with suspensions of *V. cholerae* in sterilized sea-water, even found that a two-hours' exposure of these specimens to sunlight in boxes covered with transparent or with cobalt glass was sufficient to kill the vibrios.

Other rays

Schiavone & Trerotoli (1913; see also Galeotti, 1916) found that cholera vibrios, if suspended in saline and exposed in glass dishes in thin layers (2-3 mm) to the rays of a mercury vapour lamp 20 cm distant, were killed in one minute, even though the temperature did not exceed 20°C. Under the same conditions the vibrios in blood serum were killed in half an hour, those in broth or urine in 2 hours, and those in milk in 2½ hours. Pieces of material soaked with suspensions of *V. cholerae* were rendered sterile by the ultraviolet rays of the lamp in 15 to 45 minutes.

As shown by Rieder (1898) exposure of cholera vibrios to X-rays for 20-30 minutes is apt to inhibit their growth or even to kill the organisms.

Action of supersonic waves (Fig. 20)

As established by Violle (1950), the action of supersonic waves on saline suspensions of *V. cholerae* led as a rule to complete lysis of the organisms. Since, however, supersonic irradiation was capable of killing the vibrios without visibly changing them, stained preparations of treated suspensions were apt to show all transitional stages from typical organisms to complete disappearance. A further interesting observation was that, as shown by tests with methyl red, the irradiation led to a change of the pH of the vibrio suspensions, the yellow-orange colour of the suspensions changing after treatment for about ten minutes to red and then to pink, and finally quite disappearing.

Acids

As alluded to earlier in this chapter, *V. cholerae* is extremely sensitive to the action of acids. In this connexion, Kolle & Schürmann (1912) stated
that hydrochloric acid or sulfuric acid kills the vibrios in a few seconds if used in a concentration of 1/10 000, and lactic acid produces this effect even in weaker concentrations.

In the course of a study on the viability of the cholera vibrios in milk curd, which will be referred to later, Panja & Ghosh (1945) found that, besides hydrochloric acid and lactic acid, acetic acid was also immediately fatal for these organisms if present in peptone water at a concentration sufficient to produce a pH of 4.4. However, the vibrios were capable of surviving for five minutes if, instead of these acids, citric acid was used under analogous conditions.

Interesting observations on the action of gastric juice on the cholera vibrio have been made by Napier & Gupta (1942). Whereas the vibrios added to a specimen of gastric juice taken from a patient who suffered from hyperacidity were killed immediately, the organisms were apt to survive up to 24 hours (in one sample even up to 264 hours), if gastric juice from a patient with hypochlorhydria was used for analogous tests. Generally speaking, the vibrios survived for considerable periods (24 hours to maximally 370 hours) in specimens of gastric juice from which free hydrochloric acid was absent (pH 6.0-8.0) but succumbed immediately if the pH of the gastric juice was less than approximately 4.75, owing to the presence of free acid. However, since Napier & Gupta found that addition of distilled water to the specimens tested prolonged the life of the vibrios in spite of a high initial acidity, they believed that cholera vibrios ingested with a copious draught of water might pass the stomach in viable form even though large amounts of free hydrochloric acid were present. As pointed out by Greig (1929), when referring to earlier observations on the adverse action of gastric juice on V. cholerae, vibrios enclosed in a mass of food might also pass the stomach unharmed.

It may be conveniently added that, as found by Dawson & Blagg (1948, 1950), in addition to normally acid gastric juice the saliva of healthy persons appears to exert an antibacterial action on V. cholerae and might thus form a first line of defence against a not too massive infection.

Disinfectants

As was early noted by Koch (1885) and confirmed by ample later observations, the usual disinfectants, even if used in low concentrations, exert a rapidly lethal action on V. cholerae in suspensions or fluid cultures and inhibit the growth of this organism if added in small amounts to solid media destined for its cultivation. Thus Koch and his co-workers observed that phenol (carbolic acid), if used in a concentration of 0.5% killed the vibrios in 10 minutes, while at a concentration of 1%, the organisms were killed in 5 minutes. Babes (1885) found that mercury perchloride, if added to gelatin at a concentration of 1/15 000, prevented the growth of the cholera vibrios, while according to Forster (1893) these organisms were killed...
within 5-10 minutes if incorporated in mercury perchloride dilutions of 1 in 2 or 3 millions.

Other antiseptic substances

As will be gathered from the summaries of Kolle & Schürmann (1912) and Mackie (1929) as well as from more recent publications, in addition to the usual disinfectants a considerable number of other substances endowed with antiseptic properties have been found to exert an inhibitory or lethal action on *V. cholerae*. The following deserve mention:

Soap. The conclusions reached by Jolles (1893) that various sorts of soap were endowed with vibriocidal power were not confirmed by Murillo (1912), in whose experience addition of soap to nutrient media even in a concentration of 1/10 did not inhibit the growth of *V. cholerae*. Kolle & Prigge emphasized, therefore, that “even most thorough washing with soap was incapable of destroying the cholera vibrios”.

Alcohol. As maintained by Babes (1885), the maximal concentration at which alcohol added to nutrient media did not inhibit the growth of *V. cholerae* was 1/15. It is in accord with this observation that, as established by Van Ermengem (1885), broth cholera cultures became sterile within half an hour, if absolute alcohol was added at a proportion of 1:10.

Iodine. In the experience of Babes (1885), addition of iodine to nutrient media at a concentration of 1/600 to 1/800 was just incapable of inhibiting the growth of *V. cholerae*. Bujwid (1892) found that iodine vapours retarded the growth of this organism but established, in accord with previous experiences of Neisser (1887) and Riedlin (1888), that iodoform exerted a far more marked action in this respect. Since various cholera-like vibrios tested by Bujwid with iodoform were far less inhibited in their growth than *V. cholerae*, he suggested that this fact might be used in differential diagnosis—a proposal which is now interesting merely from the historical point of view.

Potassium permanganate. In contrast with the statement of Babes (1885) that potassium permanganate did not inhibit the growth of *V. cholerae*, Panja & Ghosh (1943) maintained that this chemical was lethal to cholera vibrios and still more to cholera-like vibrios and that, therefore, “fruits and vegetables artificially infected with cultures of *V. cholerae*... can be effectively disinfected by soaking them in permanganate solutions of 1/5000 to 1/10 000 dilutions for 5 minutes”. Since, however, this conclusion is not in accord with Babes’ observations and also not with the results of recent experiments made with other organisms such as *S. typhosa*, one should—as justly stated by the editor of the *Tropical Diseases Bulletin* (1943)—be cautious in accepting the recommendation of Panja & Ghosh until their results are confirmed by further tests.
Copper sulfate. Copper sulfate, found effective against *V. cholerae* in a concentration of 1/600 by Van Ermengem (1885) and in higher dilutions by Babes (tolerance limit 1/3000-1/5000), was recently again recommended by Halawani & Omar (1947) who found that this compound “is lethal in dilutions ranging from 20-45 parts per million to *Vibrio cholerae* in concentrations ranging from 10 to 1,000 million per cc. of Nile water.”

It is of interest to add that Bose & Chakraborty (1948) found metallic copper to be vibriocidal. In the presence of strips of copper foil, *V. cholerae* could not be recovered from suspensions in distilled water, in which the vibrios normally survived for two days. In filtered tank water, the presence of copper foil shortened the life of the organisms to about 1 1/2 hours as against a survival for 15 days in the controls.

Water which had been in contact with copper foil for periods of two or four hours also proved bactericidal within 30 minutes and 15 minutes respectively if cholera vibrios were added subsequent to the removal of the metal. However, no bactericidal effect was noted if water which had been in contact with copper foil for 48 hours was used for the preparation of peptone water or Douglas broth, cholera vibrios cultivated in such fluid media remaining viable for 15 days.

Since satisfactory results were also obtained in tests carried out without the use of copper foil in polished copper vessels, Bose & Chakraborty recommended the use of these containers during cholera epidemics for the temporary storage (4-6 hours) of water. It is important to note in this connexion that chemical tests with the water samples used in these experiments failed to show the presence of copper.

### Essential oils

Since—as will be seen in Chapter 9—essential oils have been used with some success for the treatment of cholera, it is of importance to note that in the experience of some workers, such as Babes (1885) and Riedlin (1888), such oils were found capable of inhibiting the growth of *V. cholerae*. The former of these observers found mustard oil far more effective in this respect than peppermint oil, oil of cloves, bergamot oil or turpentine oil, while Riedlin (who did not test mustard oil) found turpentine oil to be most antiseptic, followed in order of efficacy first by lavender, eucalyptus and rosemary oil, then by oil of cloves. Other essential oils, including those of anise, fennel, juniper, peppermint, and thyme, were in Riedlin’s experience of “subordinate importance”.

### Aniline dyes

Shiga (1913) found that some aniline dyes, particularly methylene blue and thionin, even if used in concentrations of 1/33 000 and 1/25 000 respectively, inhibited the growth of cholera vibrios, but that by growing
them in the presence of still lesser amounts of these dyes the organisms could be made dye-fast, the strains then becoming capable of tolerating the action of the dyes at much higher concentrations.

Exploring the possibility of incorporating aniline dyes into agar media destined for the cultivation of \textit{V. cholerae}, Signorelli (1912) found that addition of dahlia, erythrosin, orcein, or safranin led to a loss of virulence of the cholera vibrios developing on such media, which became decolorized while the colonies became intensely coloured. Growth of \textit{V. cholerae} on agar containing methyl green or azolitmin also led to a decolorization of the media but, the colonies not taking up these dyes; no loss of virulence resulted.

Further interesting observations on the vibriostatic and vibriocidal properties of aniline dyes were made by Panja & Ghosh (1943), whose most important findings may be summarized thus:

(a) Brilliant green, crystal violet, methylene violet, added to agar in a concentration of 1/100 000 exerted a bacteriostatic effect on \textit{V. cholerae} and \textit{El Tor} vibrios. The same result was obtained with 1/50 000 concentrations of malachite green, acriflavin, gentian violet, methyl violet, methylene blue, fluorescein and pyronin yellowish, with thionin at a concentration of 1/25 000, with mercurochrome, safranin, and basic fuchsin in concentrations of 1/5000 respectively.

(b) Brilliant green and malachite green, if incorporated into peptone water at concentrations of 1/100 000 exerted a selective bactericidal effect on most cholera strains as well as on a large number of "paracholera" strains isolated from patients with clinical signs of choleraic disease, but these dyes did not affect the cholera-like vibrios from river water.

(c) Added in a final dilution of 1/5000, brilliant green killed the vibrios in cholera stools. These vibrios also disappeared earlier than in untreated cases from the stools of cholera patients who had been given the dye orally, but clinical improvement was not marked. Since it had been found that an excess of alkali prevented the bactericidal action of the dyes \textit{in vitro}, this comparative failure of treatment probably stood in connexion with the alkaline reaction prevailing in the intestines of the patients.

Animal charcoal

Kraus & Barbará (1915a, 1915b) claimed that it was possible to render water free from cholera vibrios by shaking it with animal charcoal or by filtering it through a layer of this adsorbent. As they finally stated, it sufficed to shake 100-ml quantities of water, which had been slightly contaminated with cholera vibrios, after addition of 1 g of charcoal for 15 minutes. However, Kolle & Prigge (1928), commenting on these findings, rather doubted that this method of treating drinking-water supplies could ensure complete sterilization.

Lime and milk of lime

Studying the action of various lime preparations on cholera vibrios, Liborius (1887) found that:

1. A watery solution, containing 0.0246% of lime was capable of destroying the organisms within a few hours.
(2) “Cholera broth cultures which contained numerous protein coagula and showed physical properties at least as unfavourable to the action of lime as cholera stools were permanently disinfected within a few hours through addition of 0.4% pure quicklime or 2% impure burnt lime in lumps.”

(3) The lime was most active if it was used in the form of powdered pure quicklime or of 20% milk of lime prepared from the latter.

Milk of lime, the vibriocidal action of which in a concentration of 20% had been confirmed by Giaxa (1890), was according to Pfuhl (1892) prescribed for the disinfection of cholera stools in an instruction (Anweisung zur Desinfektion bei Cholera) issued in 1892 by the Prussian authorities. According to this, one was advised to prepare milk of lime with 1 l of pure quicklime reduced to small pieces and 4 l of water, to add the finished product to an equal amount of the stools to be disinfected, and to let the mixture stand for at least one hour before disposal. Since, however, observers in Java claimed to have had bad results with this method in actual cholera work, Pfuhl made further investigations with fresh stools from patients in which V. cholerae abounded. He found the method effective, provided that the milk of lime was not merely poured over the stools but actually mixed with them. However, prolonged stirring was unnecessary.

**Chlorine and chloride of lime**

Making an early study of the disinfecting properties of chloride of lime, Niessen (1890) found that in pure cultures in the presence of 0.12% of this compound cholera vibrios usually became incapable of multiplication after one minute and invariably so after five minutes. Hence chloride of lime gave far more rapid results than quicklime, which, according to Liborius (1887) and Kitasato (1887-88), required at least one hour or even several hours to exert a disinfecting action.

Harding (1910), experimenting with water to which 1-2 drops of a 24 hours' cholera culture had been added per litre, concluded that most samples of contaminated water, if treated with one part of chlorine per million for 15 minutes, are apt to be free from cholera vibrios. However, if organic matter is plentiful, it is advisable to use higher concentrations, so as to leave, after oxidation of the organic matter, 0.5-1.0 parts of chlorine per million available for the purpose of sterilization.

Satisfactory experiments with chlorine compounds were made by Conor (1912), who found that cholera-contaminated samples of canal water from Tunis were freed from the vibrios if chloride of lime or chloride of soda were added to the water at the rate of 2 mg per litre and allowed to act for eight hours. Conor considered it essential, however, to utilize
freshly prepared solutions of these compounds with a content of free chlorine amounting to 30-40 per 1000.

According to Langer (1913), addition of 0.5 g of chlorinated lime (corresponding to about 0.12 g of free chlorine) per litre of water sufficed to kill cholera vibrios and other pathogenic bacteria, particularly if the disinfectant had been mixed with equal parts of sodium chloride to promote an even distribution. Similarly favourable results were also recorded by Ditthorn (1915), who worked with a proprietary chlorine preparation.

In contrast to the experiences mentioned above, Genevray (1940a) laid stress upon the fact that in peptone water the cholera vibrio was found to resist considerable doses of free chlorine, since an excess of 2 mg of free chlorine per 10 ml of the medium (i.e., 200 mg of free chlorine per litre) did not destroy it. Genevray found it pertinent to ask, therefore, what action chloride of lime, used in Indochina for the disinfection of ponds during cholera epidemics, could exert under these circumstances. To answer this question it would be certainly desirable to make further investigations on the action of chloride of lime on \textit{V. cholerae} in areas where cholera prevails.

\textit{Ozone}

Investigating the value of ozone for water sterilization, Schubert (1914) found this method preferable to that of sand filtration for rendering the water supplies free from cholera vibrios and from typhoid bacilli.

\textit{Symbiosis}

Voicing an often-expressed opinion, Kolle \& Schürmann (1912) maintained that:

"In the case of a simultaneous presence of bacteria causing decay or rapidly growing saprophytes no appreciable development of the cholera vibrios takes place under most natural conditions—indeed in most instances decay and decomposition are factors which rapidly destroy the cholera bacteria.” [Trans.]

In support of this contention Kolle \& Schürmann pointed out that, in the experience of Koch (1885) and subsequent workers, as a rule the cholera vibrios did not survive long in highly contaminated and decaying substrates such as the contents of cess-pools or sewers.

That also under other circumstances the presence of other bacterial species was apt to handicap or even to shorten the existence of \textit{V. cholerae} has been shown by (a) the observation of Rosenthal (1910) to the effect that cholera vibrios were unable to grow in milk or other suitable media in the presence of \textit{Lactobacillus bulgaricus}, which produced a markedly acid reaction; and (b) investigations by Panayotatou (1913), demonstrating in the water of the Nile the presence of four (not further identified) bacterial species which were markedly antagonistic to \textit{V. cholerae}, and thus
apparently responsible for the failure of the latter to persist under laboratory conditions in water samples from that river.

It is important to note, however, that symbiosis with other bacterial species was by no means always found to be unfavourable to the persistence of *V. cholerae*. Kabelik & Freudmann (1923) noted in this connexion that, when cultivated in peptone water together with *E. coli*, cholera vibrios grew far more luxuriantly than did *E. coli*. Sarkar & Tribedi (1953), again studying the relation between these two organisms, found that when a loopful of a cholera culture was added to a 24-hour-old broth culture of *E. coli*, and daily platings were made, during an initial period lasting from three to 14 days *E. coli* colonies only grew on the plates. Subsequently, however, cholera colonies appeared in increasing proportions, to become finally solely present. Sarkar & Tribedi found that this evolution was paralleled by changes in the pH of the broth culture: the preliminary cultivation of *E. coli* led, after 24 hours, to a lowering of the initial pH of 7.6 to 7.2. After addition of the cholera vibrios, the pH rose, vibrio colonies appearing as soon as it had reached 8.8 and being solely present, when the pH had reached 9.2. However, in the opinion of the two workers, this rise of the pH alone was not responsible for the disappearance of *E. coli* because it was found that (a) prolonged cultivation of this organism alone in broth led to a pH of 9.0, at which it was able to survive; and (b) it was viable for several days in broth with an initial pH of 9.8, which it lowered within 24 hours to 9.3. It was also noted that, while *E. coli* was unable to multiply in a broth culture in which cholera vibrios alone had survived, it could multiply in these cultures if the vibrios had been killed through heating for one hour at 60°C. Sarkar & Tribedi postulated, therefore, that the antagonistic action exerted by *V. cholerae* and (as they also established) by cholera-like vibrios on *E. coli* was due to the presence of a thermolabile colicidal substance. The antagonism exerted by the cholera vibrios was also manifest if they were present in stools together with *E. coli*.

Carrying out exhaustive and exact studies on the effect on *V. cholerae* of the concurrent presence not only of *E. coli*, but also of *Aerobacter aerogenes*, of *Proteus vulgaris*, of *Streptococcus faecalis*, a Gram positive coccus isolated from water, and of water vibrios not agglutinable with cholera-immune serum, Read et al. (1939) established that under these circumstances

"the agglutinable vibrio can survive in weak peptone water and salt solutions even when present in smaller inoculum, except in the presence of certain inagglutinable vibrios. In several experiments it survived for two weeks or more in the presence of the latter vibrios."

The observations recorded above as well as those to be dealt with now show that the cholera vibrio is by no means as invariably frail an organism as it is assumed to be by some authorities.
Viability of V. cholerae Outside the Body

Faeces

Describing the experiments made by Koch and his co-workers during their initial work, Gaffky (1887) stated that

"if intestinal contents or faeces rich in cholera bacilli but containing other bacteria as well were put on moist earth or linen and were kept in a manner preventing exsiccation, at first the cholera bacilli grew most luxuriantly so that after 24-48 hours specimens taken from the surface contained—as proved by microscopic examination—the cholera bacilli practically in pure culture. However, after a few days already they began to die and the other bacteria started to multiply."

Though Koch and his co-workers were unable to state definitely what the maximal period of survival of the vibrios under these and similar conditions was, their observations indicated that—particularly if decomposition took place and/or other bacteria were present—the V. cholerae did not persist long.

While Koch and his co-workers made their observations with what Greig (1914a) afterwards called “uncultivated” strains of V. cholerae, i.e., directly with the faeces of cholera sufferers, many of the numerous other workers investigating the survival of this organism used for their tests the dejecta of individuals free from cholera to which they added cultivated vibrios. While one must fully agree with Abel & Claussen (1895), Greig (1914a), and Gildemeister & Baerthlein (1915), who made the most valuable investigations in this field, that only observations with cholera faeces can be considered valid, it is interesting to see that they adduced different reasons why the results obtained with artificially infected stool specimens should be rejected. Abel & Claussen (1895) maintained in this connexion that quite possibly the “comma bacilli” in the cholera faeces, where they are often present in almost pure culture, possessed a higher vitality than those in artificial stool mixtures, where they were subjected to competition with other bacteria. Greig (1914a), on the contrary, recommended “uncultivated” material because experiments previously made with typhoid bacilli seemed to indicate that stock cultures of V. cholerae were more resistant than the vibrios in the faeces. Gildemeister & Baerthlein (1915) stressed with much reason the importance of results with rice-water stools because, owing to the abundance of mucus material, these were less prone to undergo exsiccation than other kinds of faeces.

Several of the early workers who could examine genuine cholera stools in Europe found that the average period of persistence of the vibrios was longer than the preliminary findings of Koch and his co-workers in India had indicated, and some reported instances of an excessively long survival of the organisms. While in view of the limitation of the differential diagnostic methods available to the early observers the latter records must be inter-

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1 The occurrence and persistence of cholera vibrios in animals (e.g., in flies and aquatic animals) will be dealt with in later parts of this book.
preted with great caution, the fully reliable findings of Abel & Claussen (1895) and of Gildemeister & Baerthlein (1915) deserve great attention.

Abel & Claussen (1895) worked with 31 cholera stools which had been sent for diagnostic purposes to the Institute of Hygiene in Königsberg in Prussia. Once the diagnosis of cholera had been established, these samples, kept in the well-closed bottles in which they had arrived, and protected against direct sunlight, were stored at room temperature (13°-16°C). Re-examinations with the aid of peptone water enrichment were made daily or at least every few days. If loopfuls of the stools gave no results, larger amounts up to 50 ml were used for peptone water enrichment and platings and only if these also proved negative, were the cholera vibrios considered to have disappeared.

Abel & Claussen found under these circumstances a persistence of the cholera vibrios for 1-5 days in eleven instances, for 6-10 days in six, up to 15 days in nine, for 15-17 days in three, for 24 and for 29 days in one instance respectively. It will be noted, therefore, that (a) the cholera vibrios disappeared from about one-third of the samples within five days and (b) that a survival of the organisms for more than 15 days was not frequent (16.1%).

Working during the First World War at Posen (now Poznan), Gildemeister & Baerthlein (1915) examined 70 stools derived partly from cholera patients, partly from supposedly healthy carriers, only few of the samples showing a typical rice-water-like appearance. Their technique differed in some details from that of Abel & Claussen, particularly because (a) they kept their specimens (protected from direct sunlight and at room temperatures ranging from 12°C to 21°C with an average of 18°C) in covered Petri dishes, so that they were apt to undergo exsiccation; and (b) they used for their platings blood-alkali agar as recommended by Dieudonné (1909), whereas Abel & Claussen had worked with gelatin plates.

The results obtained by Gildemeister & Baerthlein may be summarized thus:

<table>
<thead>
<tr>
<th>Period of survival (days)</th>
<th>Number of specimens</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>25.7</td>
</tr>
<tr>
<td>2-5</td>
<td>8</td>
<td>11.4</td>
</tr>
<tr>
<td>6-10</td>
<td>9</td>
<td>12.8</td>
</tr>
<tr>
<td>11-15</td>
<td>8</td>
<td>11.4</td>
</tr>
<tr>
<td>16-20</td>
<td>11</td>
<td>15.7</td>
</tr>
<tr>
<td>21-30</td>
<td>11</td>
<td>15.7</td>
</tr>
<tr>
<td>31</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Gildemeister & Baerthlein concluded, therefore, that

"(1) Cholera vibrios succumb in a major part of the cholera stools within a short time;
(2) However, in a not inconsiderable part of the stools they remain viable for several weeks, sometimes more than 30 days."

Greig (1914a), using for his observations on the persistence of *V. cholerae* 94 typical cholera stools freshly collected in Calcutta and applying a technique similar to that of Abel & Claussen, but taking advantage of Dieudonné as well as of agar plates, recorded the following results:
Greig concluded from these investigations that, though there was considerable variation between individual strains, "the life of the cholera vibrio outside the human host under natural conditions in India is short," and added the equally important statement that "Temperature has a powerful influence on the vitality of the cholera vibrio outside the human host. Thus as the hot season, in Calcutta from March to June advances, the life of the organism becomes shorter: in the present research the minimum duration of life was reached in June. On the other hand, from December to February, the cold season, the vitality is greater, and the maximum duration of life occurred in February. Again in August when the monsoon has fully developed and the temperature has fallen somewhat the life is longer than in the hot season but as the number of cases of cholera during August and September is small my observations during this period were fewer."

That the prevailing temperature exerts an important influence on the period of survival of *V. cholerae* in the faeces of the patients, was also shown by observations in Japan. In addition to findings recorded in this respect by Takano, Ohtsubo & Inouye (1926), Soda et al. (1936) noted that if specimens of one and the same cholera stool were kept at 37°C, at room temperature and in the ice-box respectively, survival of the vibrios was longest (up to eight days) in the last case, shortest (sometimes only 3 hours) at body temperature (37°C). Identical experimental observations had been previously recorded by Shoda, Koreyada & Otomo (1934).

These observations as well as the fine studies of Greig leave little room for doubt that differences in the prevailing temperature were largely, if not solely, responsible for the marked differences observed in regard to the length of survival of the causative organisms in cholera stools in India and Europe respectively. It is significant that analogous differences were noted when the length of survival of the cholera vibrios in sewage, cesspools or septic tanks and the like was studied. Flu (1921a) established in this connexion that, in contrast with *S. typhosa*, cholera vibrios persisted in the septic tanks of Batavia not, or not much, longer than 24 hours. In Europe on the contrary, to judge from the summary of Fübringer & Stietzel (1908), survival periods of *V. cholerae* in cesspools, manure, and the like for one to two weeks have been recorded by several observers. That in these cases
temperature differences also played an important role is suggested by observations of Ohwada (1924), which showed that *V. cholerae* survived in sewage at 37°C for one day, at room temperature for four days, and in the ice-chest for twelve days.

**Dead bodies**

Dunbar (1896), the first and, it would seem, the only worker to try to determine the length of survival of the causative organisms in cholera victims, was able to examine the dead bodies of 10 individuals who had been buried during the 1892 outbreak at Hamburg. Most of these persons had been buried in September and one at the end of December, and they had been exhumed during the period from December 1892 to April 1893. It was impossible to find cholera vibrios in any of these bodies, which included (a) one buried on 1 September and exhumed on 5 December and (b) another buried on 25 December and exhumed on 6 April of the following year. It is curious that the intestinal contents of the latter victim, obtained at autopsy and kept in the laboratory at room temperature, still proved positive for *V. cholerae* at the time of the exhumation of the body and for two weeks afterwards.

In view of these findings made during autumn and winter in Europe, it is altogether unlikely that the causative organisms survive for any considerable length of time in the dead bodies of individuals succumbing during cholera outbreaks which typically take place during the warm season in the countries usually affected.

**Contaminated material**

Besides the work of Koch and his co-workers, whose experiments with moist earth and linen were referred to above, the fate of *V. cholerae* in these and other substrates contaminated with faeces or with material from cultures has been studied by numerous other workers. While the experience they gained with foodstuffs will be dealt with separately below, the following findings obtained with other materials deserve attention.

**Earth and dust.** Nicati & Rietsch (1885) claimed that, if cholera stools were sprinkled on moist earth, the vibrios remained viable for 14-16 days. However, Uffelmann (1893b) concluded from numerous experiments that *V. cholerae*, if added at high concentration to samples of garden earth, survived at room temperature for two to three days only. The viability of the organisms could be prolonged to 12 days if the samples were kept at 6°C, and to 16 days at 0°C to +1°C, i.e., at temperatures hardly ever met with in the regions where cholera is prevalent. In fact Flu (1915) found that in Batavia, Java, the length of survival of the causative organisms in the rice-water stools of cholera patients poured on the ground did not exceed seven days even if the weather was moist.
Studying the possibilities of aerial transmission of infectious diseases, Germano (1897) exhaustively experimented with different dusts to which cholera vibrios in suspensions or incorporated in normal faeces had been mixed. He found that the vibrios survived well if the dust was kept moist but died rapidly (maximally within three days in the case of brick-dust), if exsiccation took place. Germano concluded, therefore, that the chances of transmission of cholera through the air were “extremely slight”.

It may be conveniently added that, according to the summary of Takano, Ohtsubo & Inouye (1926), investigations on the survival of *V. cholerae* on coal have been made by Hata & Matsuda (1906). These two workers noted that the organisms persisted in a mass of moistened coal for seven days, and in a mass of dry coal for 21 days, moisture apparently hastening the death of the organisms by facilitating the growth of other bacteria.

**Cloth and cotton.** A few workers, e.g., Gamaleia (1893) and Karlinski (1895) found that under highly artificial conditions counteracting exsiccation the cholera vibrios on pieces of cotton or cloth which had been soaked in suspensions of these organisms could survive for several weeks or even months. However, most observers who performed their tests under conditions comparable to those actually obtaining agree that the cholera vibrios persist on these contaminated materials for a few days at the most (one to five days according to the summary of Jettmar, 1927). The earlier observations made to this effect have been confirmed by those made during the 1947 cholera outbreak in Egypt, when, as recorded by Shousha (1948), it was shown by tests with faeces of the cholera patients that *V. cholerae* survived on depurated cotton for two days only, and on raw cotton and cloth for three days.

**Leather and rubber.** According to Gohar & Makkawi (1948), the causative organisms in the stools of cholera patients survived for two days on leather but for not more than six hours on rubber.

**Paper.** To judge from the scanty information available, the survival period of *V. cholerae* on paper or objects made of paper is short. Uffelmann (1892) stated in this connexion that if cholera stools were left to dry on the printed page of a book (which took about 10 minutes), and the book was then closed and kept in a cupboard, the vibrios survived for at least 17 hours. Under the same conditions the organisms remained viable for at least 23½ hours on letter-paper enclosed in an envelope, and for at least 20 hours on a postcard.

Germano (1897) found that the *V. cholerae* was able to survive for one day only on blotting-paper which was let to dry after it had been soaked in a suspension of the organisms. If exsiccation was prevented, the vibrios were still viable on the 20th day.

Tests with Chinese bank-notes which had been handled by fingers contaminated with cholera stools showed that, after the bank-notes appeared
to be dry, the vibrios remained alive for maximally four hours (Jettmar, 1927). However, recent observations in Egypt (Shousha, 1948) showed a survival period of *V. cholerae* on bank-notes contaminated with cholera stools for two days, and on postage stamps treated in the same manner for one day.

_Metals._ Uffelmann (1892) found that if cholera stools were put on copper and silver coins and permitted to dry the vibrios remained viable for 10-30 minutes only. Identical results were obtained with brass plates. Tests made during the 1947 cholera epidemic in Egypt with faeces-contaminated coins showed a survival of *V. cholerae* for seven hours (Shousha, 1948).

_Tobacco._ Wernicke (1892) found that even on moist cigars and snuffing-tobacco cholera vibrios succumbed within 24 hours.

_Food_ 

For obvious reasons, the fate of *V. cholerae* in food materials contaminated with cholera stools or cultures has attracted the attention of numerous workers. Babes (1885), who seems to have been the first to make systematic studies in this respect, noted that the cholera vibrios remained alive up to 48 hours on fresh non-acid vegetables, potatoes, and cheese, but not longer than 24 hours on sour fruit and vegetables.

Essential findings made by subsequent workers may thus be summarized.

_Meat._ There can be no doubt that under suitable environmental conditions meat and meat products form a favourable substrate for the survival of *V. cholerae*. Thus Uffelmann (1892) found that on roast pork, kept under a glass bell, the vibrios survived for at least eight days. Lal & Yacob (1926), testing various Indian foodstuffs, placed meat high among those found potentially suitable for the cultivation of the cholera vibrios. Japanese observers (see Takano, Ohtsubo & Inouye, 1926) found meat a suitable substrate for the survival and, during the first 20 hours after contamination, for the multiplication of the *V. cholerae*. Cholera vibrios on the surface of meat which was kept in the open during mid winter were found to be able to survive for one to two weeks.

_Fish and shellfish._ As shown by numerous observations, fish and shellfish, stored pending consumption, form a suitable substrate for quite prolonged survival of *V. cholerae*.

Systematic studies made by Friedrich (1893) showed that cholera vibrios were apt to survive on fresh fish for two days, on smoked herrings for one day (according to Uffelmann (1892) even for four days), on caviar for three to six days or, if the latter was kept in the ice-box, even longer.

Takano (1913) found that cholera vibrios smeared on fish meat, which was then kept at room temperature during the month of October, survived
for three to four days, but that storage of the material in the ice-box prolonged the viability of the organisms to 10-12 days.

As quoted by Takano and co-authors (1926), Toyama, working mainly with freshwater fish, established that cholera vibrios smeared on the meat of these animals remained viable for two to three days during midsummer, for seven to ten days in early summer, for one to two weeks in mid winter. Storage of the fish in an ice-box at 3°-8°C prolonged the survival period of the vibrios to 14-19 days, occasionally even to 25 days.

Toyama further stated that, when oysters or clams were kept in cholera-polluted sea water, the vibrios rapidly entered their gastro-intestinal tract and survived there for 1½ months at a temperature of 0°-5°C, and for 15-20 days at 22°C. When cholera vibrios were smeared on shelled oysters kept at room temperature (about 20°C), the number of organisms first decreased but soon began to increase and reached a maximum in 68 hours, followed by a gradual decrease and disappearance of the vibrios in 171 hours. *V. cholerae* remained viable for 20 days if smeared on oysters or clams which had been killed and sterilized by boiling. As shown by tests with contaminated oysters which had been soaked in dilute acetic acid, it was comparatively easy to kill the cholera vibrios on their surface, but the organisms survived in the intestinal tract of the oysters for seven hours when 1%-2% acetic acid was used, for two hours in the case of 3% acetic acid, and for 45 minutes if the concentration was increased to 4%-5%.

**Milk.** In a classical study on the behaviour of *V. cholerae* in milk, Kitasato (1889b) established the following periods of survival of the organisms:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Raw milk</th>
<th>Raw milk with 10% sodium carbonate</th>
<th>Steril-sterilized milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>36°C</td>
<td>14 hours</td>
<td>55 hours</td>
<td>2 weeks</td>
</tr>
<tr>
<td>22°-25°C</td>
<td>1-1½ days</td>
<td>Still fairly numerous after 78 hours</td>
<td>Still viable after 3 weeks</td>
</tr>
<tr>
<td>8°-18°C</td>
<td>2-3 days</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Noting an incessantly progressing acidification of the milk media in the course of these tests, Kitasato emphasized that

"the length of survival of the cholera bacteria is dependent upon the reaction of the milk; the more rapidly the milk sours, the more rapidly the cholera bacteria therein perish; however, the cholera bacteria survive until the milk becomes strongly acid." [Trans.]

Since heating of samples of raw or raw alkaline milk inoculated with cholera vibrios for five minutes at temperatures ranging from 96°C to 100°C rendered the samples sterile, Kitasato also concluded that "boiling is the simplest and most effective method to free milk from cholera germs".

Most subsequent observers confirmed that under the ordinarily prevailing temperatures cholera vibrios could survive in raw milk for at least
one to two days, regardless of whether or not the milk became acid in the meanwhile or, as some workers such as Heim (1889) and Basenau (1895) expressly stated, even regardless of whether or not the milk had curdled. It is, however, interesting to note that in the experience of Panja & Ghosh (1945) cholera vibrios added to Indian milk-curd (dahi) were killed within five minutes and that according to recent observations in Egypt (Shousha, 1948) the life-span of V. cholerae added to already sour milk was only one hour. These observations seem in accordance with experimental findings of Heinemann (1915), who established that cholera vibrios were immediately killed if added to samples of sterilized milk which contained 0.45% lactic acid or, as seems indicated by the protocols of this worker, even at lower concentrations of the acid.

It is, on the other hand, important to realize that boiled milk, if contaminated by V. cholerae pending storage, is at suitable temperatures a substrate favourable for the initial multiplication and survival of this organism. Observations made in this respect in the Berlin Gesundheitsamt (1892) showed that, whereas cholera vibrios added to raw milk survived for less than 24 hours, they remained viable for nine days in milk which had been boiled for one hour and again cooled before contamination.

Whey. According to Heim (1889), whey, even though its originally alkaline reaction had turned weakly acid, was still positive for V. cholerae on the second day following contamination.

Butter. Heim (1889) found that cholera vibrios, while surviving for a day only on low-grade, slightly acid butter, remained viable on butter of better quality for over one month. However, other observers, such as Laser (1891) and Uffelmann (1892), recorded periods of survival of V. cholerae on butter not exceeding one week.

Cheese. As stated by Babes (1885) and some subsequent European observers, cholera vibrios were apt to remain viable on cheese for 48 hours. Shousha (1948) in Egypt noted a survival period of the organisms on "white" cheese (probably a local product) for only two hours. An interesting parallel to this observation is that, according to Heim (1889), cottage cheese gave positive results only immediately after contamination with V. cholerae.

Salt. As maintained by Takano, Ohtsubo & Incuye (1926), experiments carried out in Japan had shown that the cholera vibrios do not multiply in salt solutions and are even gradually killed. The data they furnished to support this contention are summarized in Table XIX. They added that "In salting fish, if impure salt be used and left at a room temperature, the cholera vibrios survive for 2 weeks. The effect is better if the abdominal viscera of the fish be removed and the fish be packed in salt."
**TABLE XIX. PERIODS OF SURVIVAL OF V. CHOLERAE IN SALT SOLUTIONS**

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Chemically pure NaCl</th>
<th>Common cooking salt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37° C</td>
<td>Room temperature</td>
</tr>
<tr>
<td>5</td>
<td>1 month</td>
<td>1 month*</td>
</tr>
<tr>
<td>10</td>
<td>20 days</td>
<td>1 week</td>
</tr>
<tr>
<td>15 - 25</td>
<td>1 day</td>
<td>1 day</td>
</tr>
<tr>
<td>Saturated solution</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>.</td>
<td>.</td>
</tr>
</tbody>
</table>

*Autumn season

Analogous investigations by Genevray & Bruneau (1938a) showed the survival of *V. cholerae* in solutions of either NaCl or sea-salt to be as follows:

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Period of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>One month or more</td>
</tr>
<tr>
<td>5.0-7.0</td>
<td>More than three weeks</td>
</tr>
<tr>
<td>8.0</td>
<td>More than two weeks</td>
</tr>
<tr>
<td>9.0-11.0</td>
<td>24-48 hours</td>
</tr>
</tbody>
</table>

Since no multiplication of the vibrios was observable at salt concentrations exceeding 8% (80 per 1000), Genevray & Bruneau felt certain that sea-salt in bulk did not play a role in the spread of cholera.

However, though high salt concentrations exert an unfavourable influence on the survival of *V. cholerae*, Venkatraman & Ramakrishnan (1941) found 2% solutions of sea-salt (or of the impure salt obtainable in the bazaars of India) in carefully buffered saline, prepared as will be described in Chapter 7, excellent vehicles for the transmission of cholera-suspect stools to distant laboratories. In the experience of these two workers, cholera vibrios in artificially contaminated stool samples remained viable in such solutions for 62 days, while the vibrios in actual cholera stools were preserved even up to 92 days, the pH of the solutions remaining at its original level of 9.2.

**Sugar and honey.** Shousha (1948) noted that contamination of sugar with cholera stools resulted in a survival of the vibrios for three days. To judge from tests made with cultures, *V. cholerae* survived for one day only on honey.

**Bread and cakes.** As noted by Uffelmann (1892), *V. cholerae* was apt to survive for at least one day on slices of unwrapped rye bread, for up to three days on rye bread wrapped in paper, for at least one week on bread kept under a glass bell.
According to Friedrich (1893), cholera vibrios survived on pastry not longer than 24 hours but were able to persist on biscuits for periods up to four days.

**Cereals.** Observations made during the 1947 outbreak in Egypt (Shousha, 1948) showed a *V. cholerae* survival of two days on rice and lentils contaminated with the stools of patients. A much shorter survival (7 hours) was noted in tests made with cholera cultures, thus lending support to the assumption that the organisms in the actual stools are better protected against adverse conditions than those grown on artificial media.

There can be no doubt that, as summarized by Sticker (1912), rice gruel and similar dishes prepared from cereals, if kept under suitable temperatures, form a favourable substrate for the growth of *V. cholerae*.

**Potatoes.** As summarized by Sticker, cholera vibrios were apt to survive on the surface of raw potatoes for at least 48 hours. The acid reaction initially present on the cut surfaces of potatoes was unfavourable for the organisms, but in the case of some kinds of potatoes a change to an alkaline reaction could take place spontaneously which favoured the persistence or even the multiplication of *V. cholerae*. Sticker added that cold potato dishes were a favourable substrate for this organism which could multiply there without causing visible changes.

**Onions and garlic.** Contrary to the popular belief that their consumption is apt to confer protection against cholera infection, onions and garlic actually form fairly good substrates for the survival of *V. cholerae*. Tests carried out in this respect with faeces of patients during the 1947 outbreak in Egypt showed, according to Shousha (1948), the following survival periods:

- **Onions** — outside: 2 days  
  — inside: 3 days
- **Garlic** — outside: 1 day  
  — inside: 2 days

**Green vegetables.** While according to earlier experiments, such as those of Babes (1885) and Uffelmann (1892), cholera vibrios were able to persist on green vegetables for two to three days only, longer periods of survival (up to 22 days on spinach, even up to 29 days in the case of one lettuce specimen) have been recorded by Pollak (1912). Though it has to be noted that the conditions under which this worker experimented did not correspond well to those actually prevailing, he was certainly right in stressing that persistence of an adequate degree of moisture was apt to promote a prolonged survival of *V. cholerae* on green vegetables (and also on fruit).

It is important to note that cholera vibrios can survive on cucumbers, which have a mildly acid reaction, for some days—as found by Mackie &
Trasler (1922) in Mesopotamia for three days and according to observations made in the Berlin Gesundheitsamt (1892) even for 5-7 days. For, as quoted by Sticker, Hankin (1896b) not only incriminated cucumbers as being instrumental in the causation of some cholera cases in India but supported this contention by demonstrating the presence of *V. cholerae* on the cucumbers in question. There can be no doubt that the use of human manure for fertilizing cucumbers, which are often eaten uncooked, renders them potentially rather dangerous for the transmission of cholera.

**Fruit.** Systematic investigations made with a series of different fruits and berries in the Berlin Gesundheitsamt (1892) yielded rather variable results with survival periods of *V. cholerae* ranging from one hour to between three and seven days at room temperature, for somewhat shorter periods (up to four days) at 37°C. It was noted that the organisms could survive on the surface of dried European fruit for one to two days.

The results obtained under somewhat unrealistic conditions by Pollak (1912) may thus be compared with recent experiences made with cholera stools during the 1947 outbreak in Egypt:

<table>
<thead>
<tr>
<th>Kind of fruit</th>
<th>Pollak (1912)*</th>
<th>Shousha (1948)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apples</td>
<td>16 days</td>
<td></td>
</tr>
<tr>
<td>Dates</td>
<td></td>
<td>Outside: 2 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inside: 3 days</td>
</tr>
<tr>
<td>Grapes</td>
<td></td>
<td>Outside: 2 days</td>
</tr>
<tr>
<td>Lemons</td>
<td>14 days</td>
<td>Skin: 3 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inside: 1 hour</td>
</tr>
<tr>
<td>Oranges</td>
<td>10 days</td>
<td>Skin: 3 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inside: 1 hour</td>
</tr>
</tbody>
</table>

* Maximal periods observed.

b Gohar & Makkawi (1948), whose statements often do not tally with those of Shousha, asserted "that dates contaminated from outside could act as vehicles of infection for as long as four days and that the organisms could not survive long in the inside probably on account of the splitting of carbohydrates resulting in the production of acidity".

c Pollak quoted Dobroklonski (1910) to the effect that *V. cholerae*, while surviving inside the berries for not longer than 24 hours, could persist on the outside of grapes for four days, and on their stalks for even 12 days.

Since, as will be discussed later, in China at least cut melons have been found to play quite an ominous role in the transmission of cholera infection, it is important to note that according to the laboratory observations of Friedrich (1893) the inside of these fruits was an excellent substrate not merely for the survival but for the multiplication of *V. cholerae* as long as desiccation could be prevented. It has to be added, however, that according to the experience of Mackie & Trasler (1922) in Mesopotamia, cholera vibrios were able to survive for only three days on melons, which had a strongly acid reaction at all stages of ripening.
Local foods. Lal & Yacob (1926), testing the relative suitability of certain foodstuffs used in India as substrates for the growth of *V. cholerae*, recorded the following:

1. Articles containing salt and animal or vegetable proteins, such as meats, fresh fish, boiled rice, fresh milk, bazaar biscuits, oven-baked Indian bread, halwa, as well as radish, cooked greens, and water melons were specially suitable for the growth of *V. cholerae*.
2. Contrary to popular beliefs, chillies and onions did not inhibit the growth of the vibrios.
3. Sugared foodstuffs gave variable results.
4. Fats proved poor culture media, while sour articles, such as pickles and beer, were not likely to convey the infection.

Takano, Ohtsubo & Inouye (1926) recorded the following results with Japanese condiments:

“*The cholera* vibrio smeared on the body of fish is not killed in sugared vinegar for 2 hours. It survives for over 20 hours at room temperature in vinegar-bean-paste, and soy (soy-bean sauce) has even less disinfecting power than has vinegar-bean-paste. In short, it may be said that fresh fish prepared with vinegar, bean paste or soy is dangerous food during a cholera epidemic."

As found by Genevray & Bruneau (1938b) in Indochina, soy-bean milk and soya cheese, in which cholera vibrios survived for 12 hours and which were consumed on the day of preparation, were dangerous food articles at the time of epidemics. The length of survival of *V. cholerae* was less than one hour in fermented soya sauce, four to five hours in prawn paste and three to six hours in an Annamite dish (*nuoc-mam*) made from macerated fish, and since these articles were invariably stocked for some time before they were sold, it seems unlikely that they played a role in the conveyance of cholera infection.

Beverages

Babes (1885) maintained that cholera vibrios could stay alive in coffee, chocolate, and fruit juices for 48 hours, and in beer and wine for less than 24 hours. Systematic investigations made in this direction in the Berlin Gesundheitsamt (1892; see also Friedrich, 1893) gave the following results:

<table>
<thead>
<tr>
<th>Drink</th>
<th>Viability of <em>V. cholerae</em></th>
<th>Drink</th>
<th>Viability of <em>V. cholerae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Beer</td>
<td>Up to 3 hours</td>
<td>Coffee with milk*</td>
<td>8 hours</td>
</tr>
<tr>
<td>Wine</td>
<td>5-15 minutes</td>
<td>Tea (1%)*</td>
<td>8 days</td>
</tr>
<tr>
<td>Coffee (6%)*</td>
<td>2 hours</td>
<td>Tea (4%)*</td>
<td>1 hour</td>
</tr>
<tr>
<td>Coffee with chicory*</td>
<td>5 hours</td>
<td>Cocoa (1-2%)*</td>
<td>7 days</td>
</tr>
</tbody>
</table>

* Contaminated after cooling.

Panja & Ghosh (1945) found that undiluted lime juice (pH 2.8) killed cholera vibrios within five minutes, and lime juice freshly diluted with 1% peptone water (final pH 4.4) within half an hour.
Writing in 1921, Rogers stated, without furnishing an adequate reference, that

"Hankin showed some years ago that the cholera bacillus dies out of aerated water within a few days, so unless this commonly consumed fluid can be obtained from a firm who can be relied on to sterilize the water employed in its manufacture, it is safer to keep it for several days before consuming it, if cholera is about."

Making further studies on the survival of *V. cholerae* in aerated drinks, Yacob & Chaudhri (1945) found that the organisms, when added to soda-water (pH 6.8), were no longer demonstrable after two hours, and also noted an absence of the vibrios 10 minutes after blocks of cholera-infected ice had been added to samples of non-contaminated soda-water. These ice cubes had been prepared by freezing for 24 hours in a refrigerator one litre of water, into which 7 ml of a 24-hour culture of *V. cholerae* in peptone water had been incorporated previously.

**Survival in water**

**Fresh water.** While, as described in detail by Gaffky (1887), in the course of their work at Calcutta Koch and his colleagues were able to demonstrate the presence of *V. cholerae* in a tank which served as a source of water-supply in a cholera focus, they could not reach definite conclusions regarding the possibilities of survival and multiplication of cholera vibrios in water in general, because the few investigations they could make in this direction gave discrepant results.

However, ample observations made by other workers soon filled this gap. Reviewing the results these investigators had obtained, Gotschlich summarized in 1903:

(a) Sterile distilled water was not a suitable substrate for the survival of *V. cholerae*, but addition of minimal quantities of nutritive substances or of NaCl created more favourable conditions.

(b) In the experience of some observers, the cholera vibrios could survive for prolonged periods in sterilized spring or well water, according to Wolfhügel & Riedel (1886) for periods of up to a year.

(c) On the contrary, the survival of the organisms in the filtered and/or sterilized water supplied by water-works was short, e.g., 7 days in Berlin tap-water according to Babes (1885).

(d) The periods of survival observed in the case of the raw water of wells and springs varied from about one day to several weeks (up to 10 weeks according to Kruse, 1894).

(e) The length of survival of *V. cholerae* in raw river, pond or tank water also varied within wide limits, being for instance, according to Cunningham (1889), not more than four days in the water of Calcutta tanks, varying according to Uffelmann (1892, 1892b) in the port and river water of Rostock from 1 to 20 days, amounting under peculiar conditions in the deposits at the bottom of an aquarium to three months (Wernicke, 1895).

Some of the early workers clearly recognized that the marked differences noted in regard to the persistence of *V. cholerae* in different water-samples
were due either to variations in their character, particularly their varying content of organic matter (Koch, 1884), or to extrinsic conditions, especially differences in the temperature of the water (Nicati & Rietsch, 1885), or to both these factors. The great importance of the latter factor was demonstrated by Uffelmann (1892, 1893b), who found that cholera vibrios survived in the water of the Rostock (river) port for one day at 30°C, for two to three days at 20°C and for five days at 10°C. At a temperature of 6°C the vibrios were found to survive for at least 20 days in the river water and for at least 23 days in tap water.

In marked contrast with the results obtained in the case of rivers in Europe, Hankin (1896a) found that *V. cholerae* could not persist even for two hours in the raw water of the Ganges and Jumna Rivers in India. Since boiled water samples no longer proved inimical to the growth of the organisms, Hankin postulated that the raw water of these rivers exerted a vibriocidal action due to the presence of volatile acid substances.

That river water which is unsuitable for the persistence of *V. cholerae* may also be met with in other areas where cholera is apt to prevail, was shown by observations made later by Pollitzer (1934a), who found that cholera vibrios added to specimens of the Shanghai River and creek water were no longer demonstrable in 66% of the 50 samples tested, after they had been kept in the dark at room temperature for 24 hours. A survival of 24 hours was noted in 26% of the samples, longer periods of survival up to maximally four days being exceptional. There was no doubt that the short persistence of the vibrios was due to the heavy contamination of the Shanghai surface-waters with various bacteria. Cholera vibrios added not only to sterilized but also to Seitz-filtered specimens of the waters used for the above-mentioned tests persisted for months, and in some instances almost a year.

Panayotatou (1913) considered the water of the Nile River in Egypt, in which in contrast to many rivers in other parts of the world no cholera-like vibrios could be found, as rather unsuitable for the persistence of *V. cholerae*. As noted before, she ascribed this unsuitability (which was only relative in degree, as the vibrios were found capable of surviving in raw Nile water for periods ranging from 1 to 13 days as against at least 30 days in boiled or sterilized samples) to the presence of bacteria antagonistic to the cholera vibrios. Gohar & Makkawi (1948), recently finding that when cholera stools were added to crude Nile water, the vibrios survived for four days only as against nine days if pure cultures of *V. cholerae* were used for such tests, ascribed the difference to an inimical action of the bacterial species normally present in the faeces.

Further observations on the vitality of cholera vibrios in the water of India may be summarized as follows.

Jolly (1926) raised an interesting point when drawing attention to observations which showed "the occurrence of a change in the reaction
of Ganges river-water from alcaline to acid beginning about April before
the onset of the rains, and swinging back from acid to alcaline in October
or later". Since the two waves of cholera in Eastern Bengal and Assam
coincided with the "neutrality points" twice reached by the pH values
of the Ganges water, Jolly suggested that a causal connexion might exist
between the two phenomena, the infection dying out "rapidly at times
when the reaction of the water is either too acid or too alcaline for the
organisms and when the temperature is also unfavourable".

Though it is difficult to share Jolly's belief that an alcaline reaction
of the Ganges water might have proved inimical to *V. cholerae*, one must
admit that the presence of an acid reaction of the water might have played
a role, in addition to the prevalence of temperatures unsuitable for the
survival of the organisms.

Khan & Agarwal (1929), comparing the survival of cholera vibrios
in unboiled and boiled samples of the Ganges and Jumna water, recorded
the following results:

<table>
<thead>
<tr>
<th>Kind of water</th>
<th>unboiled (days)</th>
<th>oiled (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganges</td>
<td>1 ± 0.62</td>
<td>2 ± 0.46</td>
</tr>
<tr>
<td>Jumna</td>
<td>1 ± 0.62</td>
<td>7 ± 1.97</td>
</tr>
<tr>
<td>Well water</td>
<td>1 ± 0.47</td>
<td>3 ± 0.86</td>
</tr>
</tbody>
</table>

Comparing the results which they obtained in the four individual
sets of tests they made during a period lasting from February to April,
Khan & Agarwal noted that the duration of life of *V. cholerae* in their
samples became shorter hand in hand with an increase in temperature and
absolute humidity. They recorded in this connexion that at the time of
their first experiment the mean temperature was 73°F (approximately 23°C)
and the mean average humidity 0.388, whereas the corresponding figures
for the third set of tests were 86°F (30°C) and 0.607.

In a further study Khan (1930) tried to determine to what cause the
supposed vibriocidal action of the Ganges water was due. He could find
no evidence of the existence of volatile vibriocidal substances, noting
in particular that water samples heated under conditions which would
have prevented the escape of volatile substances no longer exerted an un-
toward influence on the survival of *V. cholerae*. Khan ascribed, therefore,
the apparent loss of vibriocidal power, occurring already when the samples
had been heated for half an hour at 55°C before they were used for tests
with *V. cholerae*, to the fact that during the process of heating the bacteria
naturally present in the water were killed. Thus, instead of competing
with the cholera vibrios for the available food material, as they did in raw
water, they furnished in their dead bodies additional nutritive substances
for the vibrios. It served as corollary for this assumption that (a) in Ganges
water samples, which had been passed through Chamberland filters, the
cholera vibrios, though persisting longer than in raw water, died markedly
more rapidly than in heated water samples, apparently because the filtered
water was less rich in food materials than the heated water; and (b) in the
experience of Khan as well as of most other observers sterile distilled water
was an unsuitable substrate for the survival of *V. cholerae*.

D'Herelle, Malone & Lahiri (1930), testing various Indian water samples
(mainly well water, no river water), found that the period of survival of
*V. cholerae* in these specimens was short, as a rule not exceeding 24 hours, and
lasting maximally two to four days. They stressed the fact that the life-span
of these organisms appeared to be much shorter in India, where cholera was
prevalent, than in Europe, which was usually free from the infection.

Studying once more the viability of *V. cholerae* in certain waters of
India, Lahiri, Das & Malik (1939) recorded the following findings:

<table>
<thead>
<tr>
<th>Kind of water</th>
<th>untreated</th>
<th>autoclaved</th>
<th>raw</th>
<th>autoclaved</th>
<th>Condly-filtered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hill spring</td>
<td>1 hour</td>
<td>18 hours</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcutta tap-water</td>
<td>18 hours</td>
<td>24 hours</td>
<td>2 days</td>
<td>12 days</td>
<td></td>
</tr>
<tr>
<td>Hooghly River</td>
<td>18 hours</td>
<td>3 days</td>
<td>2 days</td>
<td>2 days</td>
<td></td>
</tr>
<tr>
<td>Tanks*</td>
<td>up to 72 hours</td>
<td>up to 12 days</td>
<td>7 days</td>
<td>18 days</td>
<td></td>
</tr>
</tbody>
</table>

* With a high salt content and rich in organic matter.

Lahiri, Das & Malik were inclined to ascribe the considerably longer
survival of the cholera vibrios in their autoclaved samples to a break­
down of suspended organic matters facilitating the nutrition of the cholera
vibrios.

It should be noted that further tests with Hooghly river water carried
out in 1942 under the auspices of the Indian Research Fund Association,
showed somewhat longer periods of survival of *V. cholerae*, amounting to
three to four days in the case of raw water, to periods up to three weeks in
the case of specimens which had been passed through L₃ Chamberland

candles.

In the course of their investigations on the growth and survival of
*V. cholerae* in water, Read et al. (1939), to whose important work attention
has already been paid above, did not test raw water samples. However,
they carried out tests with autoclaved samples of water from Calcutta
tanks, in which they noted survival periods of the cholera vibrios ranging
from about five to over 30 days. A general conclusion reached by these
workers was that

“Available figures of analyses of natural waters in the Calcutta area suggest that the
requisite conditions for multiplication and survival [of *V. cholerae*] as far as salt content
and organic matter are concerned are present in most of the natural sources.”

Read et al. also adduced evidence to show that there was a relationship
between the prevalence of cholera and a high monthly average of total
solids in the Calcutta waters.

Since the important observations of Read & Pandit (1941) on the per­
sistence of *V. cholerae* in the natural waters of rural areas in India are of
epidemiological significance rather than being germane to the subject of bacteriology, they will be discussed in a later part of this book.

Sea water. Observations on the survival of *V. cholerae* in sea water seem to have been made first by Nicati & Rietsch (1885) who, as quoted by Gotschlich (1903), succeeded in isolating cholera vibrios from the much-contaminated sea water of Marseilles, France, harbour and establishing that these organisms could survive in sterilized sea water for periods up to 81 days.

Jacobsen (1910), working with water from the Copenhagen, Denmark, harbour (salt content varying from 8.9 to 16 per thousand), found that in this substrate cholera vibrios persisted for 7-17 days during August and September, up to 47 days in November and December, and ascribed this difference to a higher microbial content of the water during warm weather.

Comparing the vitality of *V. cholerae* in artificially contaminated samples of water from the bay of New York and from the Atlantic Ocean with that in two kinds of tap-water (Brooklyn and Staten Island), Gelarie (1916) recorded the following results:

<table>
<thead>
<tr>
<th>Kind of water</th>
<th>Survival in days (at room temperature in the dark)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bay water</td>
<td>7-47</td>
</tr>
<tr>
<td>Ocean water</td>
<td>7</td>
</tr>
<tr>
<td>Tap water</td>
<td>1 or 3</td>
</tr>
<tr>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

* The organisms were still viable at the end of these observation periods.

Long periods of survival of *V. cholerae* in artificially prepared sea water were recorded in 1933 by Yasukawa (22 days at the surface and 30 days at the bottom of a tank) and in 1939 by Venkatraman (at least 74 days survival in 2% salt water). On the other hand, Flu (1921) noted a survival of the vibrios in the water of the port of Batavia, Java, for four days only, while Gohar et al. (1948), working with samples from some Egyptian ports, found that the vibrios disappeared from raw sea water in about 24 hours. A moderately long persistence of the organisms in sea water was noted by some early Japanese observers quoted by Takano and co-authors (1926) thus:

<table>
<thead>
<tr>
<th>Author</th>
<th>Length of survival of <em>V. cholerae</em> in sea water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nogami (1902)</td>
<td>Raw sea water: 3-4 days at 37°C, 9-10 days in the ice-box. Sterile sea water: 30-42 days at 37°C, 53-65 days in the ice-box.</td>
</tr>
<tr>
<td>Yano, Okazaki &amp; Hiromi (1904)</td>
<td>At 37°C, 12 days in raw and 83 days in sterile sea water; at room temperature, 26 and 152 days respectively, in a dark room at room temperature, 44 and 209 days respectively, in the ice-chest (3-8°C) 27 and 230 days respectively.</td>
</tr>
<tr>
<td>Matsuda (1920)</td>
<td>7-10 days in raw sea water not exposed to direct sunlight, depending upon the degree of contamination of the water. Exposure to direct sunlight rapidly killed the vibrios.</td>
</tr>
</tbody>
</table>
Remarks. (a) According to Yasuhara (1926), whom Takano and colleagues could not quote, cholera vibrios survived during winter for 11 days in the sea water of Katsuura port, for 8 days in the water of the river mouth, for 11 days in the river water.

(b) Some of these workers, carrying out parallel tests with fresh water, found that here also survival of *V. cholerae* was considerably longer at lower than at higher temperatures.

Concluding remarks. The evidence adduced above makes it clear that the chances of a survival of cholera vibrios in water depend upon an interplay of a number of variable factors, such as the temperature and pH of the water, its salt content, the amount of organic matters present, and the degree of bacterial contamination. There is little room for doubt that, provided conditions for the subsistence of *V. cholerae* do exist, the temperature of the water, which in turn depends upon the prevailing season, is one of the main factors, perhaps the principal factor, determining the length of survival of the organisms.

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Chapter 4

PROBLEMS IN IMMUNOLOGY*

Toxin Production

Dealing with the cholera problem at a conference held in 1884 at Berlin, Koch considered this disease essentially as a toxicosis, caused by a "poison" which the causative organisms excreted. However, the experimental evidence soon procured by some workers was not in favour of the presence of such an exotoxin. Thus Nicati & Rietsh (1884), the pioneers in this field, found the filtrates of young broth cultures of *Vibrio cholerae* incapable of producing signs of toxicosis in dogs. However, an intoxication, characterized by vomiting, dyspnoea or general depression, and paralysis of the extremities, could be produced in dogs injected intravenously with the filtrates of cholera cultures a week or more old, and led in some of the animals to death within 12 hours.

Further classical experiments by Cantani (1886) showed that intraperitoneal injection of dogs with peptonized broth cultures of *V. cholerae*, which had been sterilized by heating at 100°C, produced in these animals severe, though passing, signs of intoxication not dissimilar from those observed in human cholera. Subcutaneous injection of dogs with the same material led to less marked signs, that of peptone-free broth cultures was without effect—a difference due in Cantani's opinion to a more rapid growth as well as a more rapid death of the vibrios in peptonized broth. Generally speaking, this worker ascribed the appearance of the toxic signs he could produce in the above-described manner to the action of an endotoxin of the *V. cholerae* which, set free after the death of the organisms, acted in a manner comparable to that of mushroom poisons.

The assumption of Cantani that the cholera vibrios, while not secreting an exotoxin, contained a potent endotoxin was confirmed by observations of some other early workers, particularly the profound studies of Pfeiffer (1892, 1894b; see also Pfeiffer & Wassermann, 1893) and of Gamaleia (1892a).

*This chapter was written jointly with W. Barrows, M.D., Professor of Microbiology, University of Chicago, Chicago, Ill., U.S.A.
Summarizing the results of his initial investigations, Pfeiffer (1892) stated that

"quite young, aerobically cultivated cholera growths contain a specific poisonous substance which exhibits extremely toxic effects. This primary cholera toxin stands in a very close relationship to the bodies of the organisms, forming perhaps an integral constituent of them. The toxic substance undergoes apparently no change if the vibrios are killed with the aid of chloroform, thymol or through drying.

"Absolute alcohol, concentrated solutions of neutral salts, boiling heat decompose the poisonous substance, leaving behind secondary toxins which exert a similar physiological action but produce an identical effect only in a 10- to 20-fold dose." [Trans.]

It should be noted in this connexion that, according to Pfeiffer (1894a), in the case of the primary toxin obtained from chloroform-treated cultures of V. cholerae, doses varying from 2.5 mg to 5 mg per 100 g of body-weight of the experimental animals were necessary to produce death in intraperitoneally infected guinea-pigs.

As described by Pfeiffer (1892), the most conspicuous sign of cholera intoxication in these animals was an incessant drop of their body temperature which, apt to commence as early as 1½ to 2 hours after the toxin administration, became most marked (as low as 30°C intrarectally) in fatal cases. Hand in hand with this temperature drop the animals became prostrated; their hind extremities became paralysed, and fibrillary convulsions of the musculature could be observed. If a sufficiently high dose had been given, death usually occurred after 12-16 hours. Administration of lesser doses led to a less marked drop of the body temperature (e.g. to 34°C) and, though apt to show serious signs of intoxication, the animals became well after 24 hours.

Gamaleia (1892), whose independent work fully confirmed the findings of Pfeiffer, came to the conclusion that the labile primary cholera-toxin was a "nucleo-albumin" which became converted through exposure to temperatures above 60°C or to strong chemicals into a more stable "nuclein". What relationship exists between this substance and the "nucleoprotein" of the cholera vibrios prepared by Galeotti (1896), and considered by this worker to be the endotoxin of V. cholerae, is difficult to decide. It is important to note in this connexion that comparative tests made by Bürgers (1910) with (a) cholera agar cultures treated according to Pfeiffer's methods to obtain the primary endotoxin, and (b) growths heated to high temperatures and consequently supposed to contain only secondary toxins, failed to show as marked differences as had been found in corresponding experiments by Pfeiffer and Gamaleia.

It is of historical interest to note that the statements made by Pfeiffer and Gamaleia met at first with considerable opposition, several workers (enumerated in the summaries of Kolle & Schürmann, 1912, Kolle & Prigge, 1928, and Kraus, 1929) maintaining that the pathogenic action of V. cholerae was due not to an endotoxin but to the secretion of a soluble exotoxin.
by the living organisms. However, as convincingly shown by Kolle & Prigge (1928), these claims, which as a rule were based upon tests with only one or a few strains—quite often of a rather doubtful nature—deserve no credence. Thus, as recently stated by Wilson & Miles (1946), it is now generally accepted that

"the cholera vibrio does not secrete a true soluble exotoxin but that it contains endotoxins which are liberated on the autolysis of the bacilli in culture or on the active disintegration of the bacilli by the cells of the animal body. The analogy that it presents with the meningococcus—another organism that readily undergoes autolysis—is very close, though the cholera vibrio is far more toxic."

Recent observations on the toxin of *V. cholerae*, as far as they fall within the scope of the present disquisition, may be summarized as follows.

Boivin et al. (1934) reported that they had been able to obtain from various Gram-negative bacteria including the *V. cholerae* through extraction with trichloracetic acid in the cold an opalescent fluid, which gave specific precipitation reactions with immune sera prepared against the organisms in question and which through prolonged boiling with weak acetic acid could be split into (a) a nitrogen- and phosphorus-containing precipitate, and (b) a specific polysaccharide which remained in solution. Further studies of the "glucido-lipoid complex" obtainable through extraction with trichloracetic acid led Boivin & Mesrobeanu (1935, 1936) to the conclusion that this compound, containing the principal part of the endotoxin of the organisms in question, as well as their somatic antigen, represented their "complete antigen", whereas the specific polysaccharide, because it was incapable of producing antibodies and antitoxins upon injection into rabbits, corresponded to a residual antigen or hapten. The two workers maintained in this connexion that, when the organisms had become rough, i.e., devoid of their specific somatic antigen, they were endowed only with a feeble toxicity due to the bacterial proteins. The latter, representing the "acid-insoluble" part of the endotoxin, were of little importance in determining the toxicity of the smooth organisms, due principally to the acid-soluble portion of the endotoxin as characterized above.

As summarized by Burrows (1944, see also Burrows et al., 1946), the findings of Boivin and his co-workers were confirmed by several subsequent observers, such as Raynal, Licou & Feissolle (1939), Damboviceanu & Barber (1940), and also by Gallut (1943), to whose work reference is made below.

A method of separating the cholera toxin from the vibrios without the use of chemicals was described by Banerjee (1942), who for this purpose took advantage of the technique of cultivation in Cellophane bags devised by Gildemeister & Neustat (1934). Slightly modifying the procedure of these workers, Banerjee used
"A long cylinder in which 2 cellophane sacs are placed, each tied tightly with string to a tube open at both ends. The length of the tubes are adjusted so that they project through the opening of the cylinder. The open ends of the glass tubes and the cylinder are plugged with cotton wool. 200 c.c. of Ramon's medium [1] is put in the cylinder outside the sacs and 50 c.c. of the same medium is put in the sacs through the glass tube. This is then sterilised in the autoclave and dialysis was allowed to proceed overnight at room temperature. In another similar apparatus oil of vaseline is put in the cylinder and also in the sacs to serve as anaerobic culture."

The culture medium in the bags was inoculated in situ with one-fifth of a slant of a 24-hour-old cholera culture and the apparatus was incubated at 37°C for 18 hours. The Cellophane bags were then removed from the cylinders and snipped with scissors so as to empty their contents.

As was established in the course of Banerjee's work, equally good growth could be obtained if the Cellophane bags were filled with normal saline instead of Ramon's broth, because satisfactory diffusion from the medium surrounding the bags took place.

To remove the vibrios from the cultivation fluids, high speed centrifugation for 20 minutes was used. Then, since the supernatant fluid was not quite free from vibrios, a current of air charged with a mixture of toluol and chloroform was passed through it.

Testing the toxicity of the fluids thus obtained, Banerjee found that—regardless of whether aerobic or anaerobic cultivation had been used—all guinea-pigs injected intraperitoneally with amounts of 2 ml died within 24 hours, whereas nearly all receiving 1 ml succumbed within 4 days. The minimum lethal dose for intravenously infected mice was 0.25 ml.

A rapid method for obtaining cholera toxin was described by Bernard & Gallut (1943a) thus:

The broth medium used by these two workers was that recommended by Ramon (1933) for the production of highly potent diphtheria toxin.

In 20 ml of this broth, to which 5 gm of glucose and of sodium acetate had been added per litre, cholera vibrios harvested from three 18-hour-old agar cultures in Roux bottles were suspended, i.e., a quantity corresponding to the weight of 8-10 mg of desiccated organisms per ml. The suspension was then kept at 37°C. To the portions removed from it for the purpose of testing, toluene was added and prolonged centrifugation was then used to obtain a fluid free from vibrios.

It was found that toxin began to appear in the suspension after 3 hours and reached a maximum after 4 hours' incubation, when the lethal dose of the centrifugate was 0.25 ml for a guinea-pig weighing 250 gm and 0.05 ml for a 15-g mouse respectively, upon intraperitoneal administration.

A still more potent toxin could be obtained by using Ramon's broth in quantities of 250-500 ml, centrifuging it after 4 hours' incubation at 37°C so as to remove the vibrios, then restoring the original glucose content (5 per 1000) and the original pH (8.0) and

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1 As summarized in the Bulletin of Hygiene (1933), Ramon's medium was prepared as follows:

One litre of water and 10 cc of pure R. E. C. I. is added to 225 gm. pig's stomach which is allowed to digest at 45°C. for about 2 hours. 325 gm. of minced veal is then added and the product is stirred vigorously, and then heat for 20-22 hours of digestion heat for 20 minutes at 80-100°C. Pass through a bag-filter. Adjust to pH 8 with NaOH. Heat to 100°C. for 20 minutes. Filter through paper. Fill out in litre quantities. Autoclave at 108-110°C. for 45 minutes. Add glucose 1.5 to 2 gm. per litre and sodium acetate 5 to 10 gm. per litre. The glucose can be added before sterilization and the acetate (in the sterile condition) afterwards, or vice versa the acetate first and glucose afterwards.
again implanting cholera vibrios at the above-mentioned rate. By repeating these operations 10 times, a toxin was obtained, the lethal dose of which for guinea-pigs of 250 g was 0.05 ml. However, quite often the toxicity became attenuated, or even altogether disappeared, after the 5th addition of fresh vibrios. Nevertheless, as pointed out by Bernard & Gallut, even without resorting to cumulative procedures it was possible to obtain with their new method within four hours a cholera toxin, the potency of which was at least equal to that of the toxin produced by vibrios cultivated in the usual manner for 7 days in Ramon's broth without glucose.

The two workers added in a second note (1943b) that in the course of cholera toxin preparation according to the above-described method, 99.5% of the vibrios were found to be dead after an incubation of four hours, when the pH of the medium had fallen to 5.8. In the opinion of Bernard & Gallut,

"the wholesale [massive] death [of the vibrios] under the influence of a pH of 5.8, due to the fermentation of the glucose, appeared to be the dominant factor in the rapid diffusion of the cholera toxin under the conditions of our experiences". [Trans.]

Carrying out comparative studies with (a) the glucolipidic substance obtained from smooth cholera vibrios according to the method of Boivin & Mesrobeanu (1936) and (b) the toxin extracted after four hours according to the procedure of Bernard & Gallut (1943a), Gallut (1943) found that

1. The content in glucolipoids in the toxin prepared according to Bernard & Gallut was higher than that obtainable from the smooth cholera vibrios themselves with the aid of Boivin & Mesrobeanu's method, usually twice as high.

2. As suggested by a positive biuret reaction, the toxin prepared according to Bernard & Gallut contained besides the glucolipidic complex also a variable quantity of proteins and polypeptides, which could be separated from the fluid containing the non-dialysable glucolipidic complex with the aid of dialysis through highly permeable membranes.

In order to confirm whether the glucolipidic complex of the toxin was different from the glucolipidic substance extracted from the vibrios with trichloracetic acid, Gallut & Grabar (1943b) resorted to comparative precipitin tests with a serum which had been obtained by the immunization of rabbits with the latter substance (see Gallut & Grabar, 1943a). The conclusion reached by the two workers on account of these tests was "that at an early stage of its elaboration the toxin contained a more complex antigen which was afterwards split into a more simple glucolipidic compound (like that extracted from the vibrios themselves) and a substance not precipitable with the immune sera".

Further studying the cholera toxin with the aid of ultrafiltration, Gallut & Grabar (1945) confirmed that the toxin consisted of two different substances—namely, (a) a simpler glucolipidic antigen and (b) a toxic substance which, because of small molecular size, could pass the ultrafilters. The two workers thus characterized the differences existing between these two substances:
More detailed studies of the hypothermy-producing component of the cholera toxin by Grabar & Gallut (1945) rendered it likely that, contrary to the previous beliefs of these workers, the substance, because found to be non-precipitable by trichloracetic acid or by sodium tungstate, was probably not of a proteid nature. The hypothermy-producing substance was found to be thermolabile and to possess no antigenic power (see also Gallut & Grabar, 1947).

Burrows (1944) used the following methods to isolate the endotoxin of V. cholerae: (a) extraction in the cold with M/2 trichloracetic acid followed by dialysis of the neutralized extract through Cellophane; (b) disintegration of the vibrios by high-speed grinding with sand, followed by centrifugation so as to separate the cellular debris from the toxic opalescent supernatant; (c) solution in 6M urea; (d) digestion with pepsin for 3-5 days, followed by removal of the insoluble material through centrifugation; (e) extraction of lyophilized vibrios with methyl alcohol, ethyl alcohol, chloroform, or ethyl ether in a Soxhlet apparatus.

The addition of 3-5 volumes of ethyl alcohol to the trichloracetic acid extract resulted in the appearance of a flocculent precipitate (found to be a polysaccharide), while the toxin remained in solution. When the filtrate from alcoholic precipitation was concentrated by evaporation, a yellow oil separated out which, containing most of the endotoxin, had a mouse MLD (minimal lethal dose) of 0.1 ml and appeared to be a mixture of alcohol and lipids, probably similar to the substance isolated from the V. cholerae with the aid of trichloracetic acid extraction by Raynal, Lieou & Feissolle (1939).

While the preparations obtained in the manner just described proved unsatisfactory because of the difficulty of separating the toxic substance from the trichloracetate and the method of direct alcohol extraction of the vibrios proved inefficient, extraction of the dry (lyophilized) vibrios with alcohol or chloroform proved highly satisfactory. The crude material thus obtained was yellowish in colour. It appeared to consist of a mixture of lipids and contained in the case of alcoholic extracts considerable quantities of inorganic salts. Purification of the crude extract could be effected by successive acetone precipitation and resolution in minimal quantities of hot absolute alcohol. The white lipid material thus obtained was negative to the Molisch, Millon, and biuret tests, and was found to contain minimal values of 5% nitrogen and 0.7% phosphorus. With proper care this
material, which represented about 2% of the dry weight of the vibrios, could be persistently prepared with a mouse MLD of 30 μg.

Burrows concluded from these investigations which, as will be discussed later, were amplified by important observations not falling within the scope of the present disquisition, that the endotoxin of the cholera vibrio was (1) stable to acids but unstable to alkali (N/10 NaOH at room temperature); (2) readily soluble in methyl and ethyl alcohols, chloroform and ether, but not in glycols; (3) readily dialysable; and (4) "closely related, possibly identical with a phospholipid".

In a preliminary note referring to further studies of the cholera endotoxin in Burrow's laboratory, Freter (1953) stated that the endotoxin of cholera vibrios grown in 1% glucose peptone water could be partially extracted in trichloracetic acid or pyridine, 70%-80% remaining in the cells. While the rapid extraction method of Bernard & Gallut gave similar results, better yields (30%-40%) could be obtained by extracting the vibrios with dilute acid at pH 3.8 for 4 hours. Freter added that "the soluble toxin so obtained could be purified by coprecipitation with calcium phosphate or carbonate, precipitation with acetone and deionization by treatment with a mixture of anion and cation exchange resins that resulted in precipitation of inactive material".

The purified substance, containing 4.5% N and 1.7% P, had an LD₅₀ of about 0.2 mg.

As further found by Freter, the residual toxicity from vibrios treated with HCl could be brought into solution by drying with acetone and further extraction at neutral pH. The toxic extract thus obtained was not soluble at pH 3.8 and no toxic material could be extracted from the precipitate at this pH. This is as well as other differences in chemical and physical behaviour seemed to indicate that the toxin of the cholera vibrio occurred in two different fractions—a conclusion which appears to be analogous to that reached by Gallut & Grabar.

Referring in greater detail to the above-mentioned investigations, Freter (1956) characterized the differential properties of the two endotoxin fractions obtained by him as follows:

<table>
<thead>
<tr>
<th>Purified acid-soluble toxin</th>
<th>Purified acid-insoluble toxin</th>
</tr>
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<tbody>
<tr>
<td>Dilute acid</td>
<td>Soluble</td>
</tr>
<tr>
<td>Chloroform-water emulsions</td>
<td>Activity in supernatant</td>
</tr>
<tr>
<td>Ion exchange resins</td>
<td>Not adsorbed</td>
</tr>
<tr>
<td>Percentage nitrogen content</td>
<td>4.5-6.0</td>
</tr>
<tr>
<td>Percentage phosphorus content</td>
<td>1.5-2.0</td>
</tr>
<tr>
<td>Reducing substance before hydrolysis</td>
<td>0</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>10</td>
</tr>
<tr>
<td>Amino-sugars</td>
<td>+</td>
</tr>
<tr>
<td>Ninhydrin test</td>
<td>-</td>
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<td>LD₅₀ (mg)</td>
<td>0.05-0.10</td>
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<th>Activity in emulsion</th>
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<tr>
<td>Adsorbed</td>
<td>12-14</td>
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<tr>
<td>1.0-1.5</td>
<td>6-7</td>
</tr>
<tr>
<td>4</td>
<td>0.35-0.75</td>
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As Freter further established, both these fractions were stable to heat (100°C for 10 minutes) and did not diffuse through Cellophane membranes; hence the acid-insoluble moiety of the toxin appeared to be different from the protein-like substance described by Gallut & Grabar (1945). The acid-soluble moiety of the toxin, on the other hand, appeared to be similar in LD₅₀, nitrogen content and solubility to the toxin fraction described by these French workers (see also Bernard & Gallut, 1945) and by Burrows (1944). Freter emphasized

"that the extraction of the toxic fractions described above did not require any steps which could not be realized in the infected human or animal body (pH 5.6 or 8.5, breaking of the bacterial cells, use of fresh vibrios, not ageing cultures). Consequently [he continued], there is no reason to believe that slightly different environmental conditions, such as might be present during the actual cholera infection, could not favor the production of endotoxin fractions with similar degrees of toxicity but with varying chemical and physical properties."

However, suggestive though these postulations and the observations referred to above in general are, in the opinion of the present authors the bulk of the evidence available in regard to the endotoxin of V. cholerae, coupled with what is known on bacterial endotoxins in general (see summary by Burrows, 1951) is more consistent with the hypothesis that the cholera endotoxin occurs as a single substance of sufficient lability to become altered by extraction and purification procedures. As a consequence, toxicity, with an original or altered pharmacological activity, might be found associated with a variety of biochemical properties, giving the appearance of several toxins and leading to divergent reports in the literature on the nature of the cholera endotoxin.

Observations on the behaviour of the better known endotoxins of the enteric bacilli, especially the dysentery bacilli, deserve great attention in this connexion. As summarized by van Heyningen (1950) and by Burrows (1951), the studies of Goebel and his colleagues have shown that toxicity lay in a relatively small basic component of the intact endotoxin of the Flexner dysentery bacillus, and that this could be prepared linked with either the polysaccharide or the polypeptide portion of the endotoxin molecule, but could not be separated in active form. Such an active moiety in this and other endotoxins was termed "TOX" by van Heyningen (1950), who suggested that the diffusible toxic substance of low molecular weight obtained by Burrows (1944) with the aid of alcohol extraction possibly represented the TOX portion of the cholera endotoxin molecule. Whether such a view on the nature of the cholera endotoxin is valid remains for further studies to determine.

Besides the observations mentioned above, several studies on the toxin of V. cholerae, which deserve attention at the present juncture, have been published by Gallut (1953d, 1954a, 1955) and by Gallut & Jude (1955).
Gallut (1953d, 1954a) reported that he had been able to study the toxigenicity of 40 cholera strains which had been isolated from 10 severely affected patients, in every instance on several occasions and usually four times. These growths, which were all of the Ogawa type and, with one exception, smooth, had been lyophilized immediately after their isolation in Calcutta and as a rule had been subcultivated once only. To determine their toxicity Gallut resorted to intraperitoneal infection of white mice. For each strain at least four groups of these animals were used, receiving respectively 0.2 ml, 0.1 ml, 0.05 ml and 0.025 ml of cholera toxin produced according to the method of Bernard & Gallut (1943a) described above. The LD_{50} for the experimental animals was calculated by the method of Reed & Muench (1938), and the titre of the toxins was determined by ascertaining the LD_{50} values per ml of the various preparations. Gallut found in this manner not only that the toxicity of the different strains varied from patient to patient but also that the growths successively isolated from each sufferer differed. While the organisms isolated early in the disease—and, more markedly still, the growths obtained at the end of the attacks—appeared to be less toxic, a "hypertoxic" vibrio, furnishing from 1.5 to 7.5 times more toxin than the other growths of the same origin, could be isolated from each sufferer in the course of the illness, on the average 60 hours after the onset of the attack. As Gallut pointed out, this particularly high toxicity of V. cholerae, becoming apparent, as it did, at about the middle of the cholera attack, could easily have remained unnoticed, since as a rule examinations were either made soon after admission of the patients to establish the diagnosis or late in the disease or in convalescence in order to ascertain whether the causative organisms were still present in the stools.

After following up these investigations with observations on the experimental virulence of V. cholerae (see below), Gallut & Jude (1955) and Gallut (1955) used the technique described above to study the relation between the toxicity of cholera strains in vitro and the temperature of incubation. Working at first with an Ogawa strain, Gallut & Jude found that

"The toxigenic power of the cholera vibrios varies according to the incubation temperature of the cultures. The most active toxins are obtained when the culture is maintained at 18° to 20°C, the most feeble at 41°. The toxins produced at 37° show an intermediate activity." [Trans.]

The two authors added that

"The cultures developed at 41°, which are feebly toxic, regain by passage at 20° merely a partial activity, inferior to that of cultures grown at 20° and even at 37°." [Trans.]

Reporting upon a continuation of these studies, Gallut (1955) stated that (a) the results obtained with the above-mentioned Ogawa strain had been confirmed through examination of other strains of the same type, and (b) identical findings had been made with one cholera strain of the Inaba type.
Making similar tests with stock strains, Gallut came to the conclusion that 

"Aging of the strains, like incubation at 41°, also diminishes the toxigenic power of V. cholerae, yet leaves the organisms with a toxicity which is not altogether negligible." [Trans.]

It was claimed by several observers, particularly by Kraus and his co-workers (see summary by Kraus, 1929), more recently also by Takita (1939) that—in contrast to the classical non-haemolytic V. cholerae—the El Tor vibrios produced in addition to an "haemotoxin" (haemolysin), as is generally accepted, also an exotoxin. However, the existence of such a separate exotoxin distinct from the haemolysin of the El Tor vibrios has been rendered rather doubtful through interesting observations of Pottevin (1913b), mentioned later, and of Gohar (1932a). Determining the haemolytic power of the supernatant fluids of El Tor broth cultures which had been centrifuged after incubation for five days and testing at the same time the toxicity of these fluids by intracutaneous injection of rabbits in analogy with the method devised by Kovacs (1932), Gohar established that toxicity appeared to run parallel with the haemolytic power of the fluids. He further found that absorption of the fluids with cholesterol, suprarenal tissue, or brain tissue, i.e., with substances rich in lipoids, rendered the fluids atoxic as well as non-haemolytic, while brain tissue extracted with ether, to remove the lipoids as far as possible, failed to produce these effects. The fluids likewise became atoxic if sheep erythrocytes had been haemolysed in them, and it was even found that

"if in a haemolysis experiment the last tubes containing the weakest dilutions and showing slight or no trace of haemolysis are tested for toxicity they are found to be nontoxic."

Gohar concluded from these observations that "the haemolysin and the exotoxin are probably one and the same thing". He admitted that old El Tor strains which had lost a great deal of their toxicity remained haemolytic. Presumably, however, this merely indicated a degradation of the toxin, the more so as it remained immunogenic.

### Virulence

Dealing in a general manner with the problem of bacterial virulence, Wilson & Miles (1946) stated that

"the term virulent is sometimes used as though it were completely synonymous with invasive; but this usage is unjustified by derivation and singularly inconvenient in practice. If rigidly adhered to it would necessitate the exclusion from the class of virulent bacteria of all of those organisms that exert their lethal effect by the production, in localized foci, of powerful toxins... It is better practice to retain the term virulent in its correct sense of poisonous, without any implication as to how the poisonous effect is produced, and to apply it to any organism which gives rise to a rapidly fatal infection."

Even apart from the fact that an invasion of the human body by the V. cholerae does not lead to a generalized infection, this broad definition
of the term "virulence" is particularly adequate for the special case of this organism which, though not producing true cholera if administered to experimental animals with the aid of the usual techniques, nevertheless is apt to cause death in these animals as well as in man, if introduced in its virulent form even in a small dosage. However, while it is legitimate, therefore, to utilize the usual animal experiments, particularly intraperitoneal injection of guinea-pigs, for an assessment of the virulence of cholera strains, it must be realized that, as far as the experimental animals are concerned, virulence defined as above is an attribute not only of the classical cholera vibrio, as is the rule for man, but also of the El Tor vibrios and even some of the cholera-like vibrios.

A further but only apparent difficulty is created by the problem of the relation existing between the virulence and the toxicity of the vibrios. Some of the early workers, considering human cholera attacks the result of a toxicosis and also bearing in mind that general signs identical with those following the introduction of living cholera vibrios could be produced in experimental animals by the administration of killed organisms or even of culture filtrates, apparently thought the terms of virulence and toxicity to be interchangeable. Dungern (1895) noted in this connexion that, as shown by the observations of Pfeiffer (1894a) freshly isolated cholera vibrios were able to multiply in the peritoneal cavity of guinea-pigs if introduced in small quantities, whereas organisms grown for prolonged periods on artificial media could do so only if administered in large doses. The question arose, therefore, whether this was due to a difference in the resistance of freshly isolated and long cultivated cholera vibrios to the bactericidal substances of the animal body or depended upon a difference in the toxicity of the strains in question, rendering them more or less able to counteract the bactericidal substances.

To answer this question, Dungern made comparative tests with (1) a freshly isolated East Prussian cholera strain so virulent that 1/8 of a loop (0.25-0.5 mg) of 20-hour-old agar cultures was lethal for intraperitoneally infected guinea-pigs, and (2) an 8-year-old often subcultivated stock culture originally isolated from a cholera patient in Calcutta which, if administered intraperitoneally in large doses (10 mg or 20 mg), produced death from toxaemia with negative bacteriological findings, the introduced vibrios having obviously been killed.

The toxicity of these two strains, tested by intraperitoneal or intravenous injection of guinea-pigs with chloroform- or heat-killed organisms, was almost exactly identical. However, while a guinea-pig intravenously injected with 2 mg of living vibrios from the recently-isolated culture showed a rapid drop of the body temperature and died in less than 24 hours, yielding abundant growths of \textit{V. cholerae} from the blood, spleen, liver, and the haemorrhagic peritoneal exudate, two guinea-pigs, injected with 2 mg of the Calcutta strain, survived, showing a passing slight drop of the temperature (to 35°C) in one instance, some fever (maximum 39°C) in the other. Dungern maintained that the dose of 2 mg was below the lethal one in the case of either strain but that obviously the virulent vibrios (East Prussian strain) were able to survive and to multiply.
The conclusion reached by Dungern on account of these experiments was "that the virulence of cholera bacilli can be quite independent of their toxicity".

The fundamental facts of cholera virulence have been elucidated through the systematic investigations of Pfeiffer (1892, 1894a, 1894b) and Pfeiffer & Wassermann (1893).

Pfeiffer established in the course of his initial work (1892) which, though carried out with a strain found afterwards to be not of a true nature, was fully confirmed by subsequent investigations with immunologically identified cholera vibrios, that in the case of living organisms the lethal dose for intraperitoneally injected guinea-pigs of about 400g body-weight was usually about one loop, occasionally as little as $\frac{1}{2}$ loop. To kill the animals by subcutaneous infection, at least 5 to 10 times higher doses were necessary. The dose necessary to cause rapid death in intraperitoneally injected guinea-pigs with chloroform- or thymol-killed cholera cultures was about three times higher than that needed in the case of living organisms—an observation supporting Pfeiffer's assumption that, though in both cases the action of the cholera toxin was the immediate cause of death, in the case of live vibrios introduced in small doses an initial multiplication of the organisms in the peritoneal cavity was an indispensable prerequisite.

Pfeiffer & Wassermann (1893) stated similarly that "in the case of intraperitoneal injection of live cultures the excess [Plus] of toxic substances which are formed through proliferation of the vibrios in of the peritoneal cavity, is most essential and, given a high virulence of the culture, can be a multiple of the amount of toxin transmitted with the originally injected bacterial substance". [Trans.]

In fact, these two workers defined virulence, as far as their investigations were concerned, "as the capability of the vibrios in question to multiply in the guinea-pig peritoneum".

As stated by Pfeiffer in a subsequent study on the etiology of cholera (1894a), he was able to continue work on the virulence of V. cholerae with numerous fresh strains isolated mostly in Germany during the recent European cholera manifestations.

According to these observations, "the cholera cultures, regardless of whether they had been derived from most severe and rapidly fatal cholera cases or from instances of slight infectious diarrhoea, showed a remarkably uniform behaviour. In the case of intraperitoneal infection, the minimum lethal dose was invariably but part of a loop (of a total capacity of 3-4 mg culture material), $\frac{1}{6}$ or $\frac{1}{8}$ of a loop usually sufficing to kill the guinea-pigs. The subcutaneously infected guinea-pigs, on the contrary, showed only a feverish reaction lasting a few hours, while pigeons (infected intramuscularly with 1 loop) survived." [Trans.]

Only three of the many cultures tested showed an aberrant behaviour, killing guinea-pigs infected subcutaneously with $\frac{1}{2}$-1 loop, and occasionally even pigeons. Such an extreme virulence of cholera cultures seemed so unusual that Pfeiffer seriously doubted the true nature of these three strains.
However, as pointed out by Pfeiffer (1894b), the virulence of strains subcultivated for prolonged periods was apt to become abated or even lost. The strain afterwards used by Dungern (see above) in particular had completely lost the ability to subsist in the guinea-pig peritoneum, vibrios injected into the peritoneal cavity of normal (i.e., non-immune) animals disappearing within 20-30 minutes without evidence of marked phagocytic activity.

Gruber & Wiener (1892), who also studied the virulence of _V. cholerae_, stressed that, especially in the case of agar cultures, only quite young (i.e., 15-30 hours old) growths were fully infectious, whereas material from cultures 48 hours old or older caused as a rule only illness of varying severity, but no death, or was even altogether inactive. That the age of the growths exerted an important influence upon their virulence seemed also indicated by the observation that intraperitoneal injection of guinea-pigs with material from the actively growing marginal portions of 48-hour-old agar cultures still proved lethal, whereas material from the centre of such growths failed to produce this effect. As claimed by Gruber & Wiener, the lost virulence of old cholera cultures could be quickly restored through subcultivation.

Fliigge (1893) and Gotschlich & Weigang (1895) were not in accord with the postulation of Gruber & Wiener that the cholera vibrios were infectious only in the stage of their youthful vigour (volleste Jugendkraft), whereas later they lost their virulence without impairment of their capability for saprophytic growth. Paralleling determinations of the number of viable organisms in cholera cultures of different age with virulence tests performed through intraperitoneal infection of guinea-pigs, Gotschlich & Weigang were able to show that in all instances, regardless of the age of the growths, one and the same number of viable cholera vibrios, approximately 200-300 million, represented the minimum lethal dose. They likewise established that by keeping their cultures at room temperature or in the ice-box instead of at 37°C, they could not only prolong the viability, but also preserve the virulence of the growths, cultures kept at lower or low temperatures for 2-3 days proving as virulent as those in the “full vigour of youth”. Gotschlich & Weigang concluded, therefore, that

“in one and the same culture the virulence of the individual viable organisms is of a constant size; the virulence of the culture is the resultant of the actions of the individual organisms; the changes of the virulence taking place in aging cultures are due solely to quantitative differences in the number of viable vibrios, not to qualitative changes taking place in the individual bacilli”. [Trans.]

Acceptable though this conclusion remains as far as recently isolated cholera vibrios are concerned, it has to be pointed out that (a) as shown already by Pfeiffer (1894b), the virulence of old often subcultivated strains was apt to become lost, and (b) as recently found, first by Shousha (1923),
and generally accepted, dissociation into the rough state leads to a decrease or loss of the virulence of V. cholerae due to qualitative changes.¹

The postulate of Pfeiffer (1894a) that no parallelism existed between the virulence of different cholera strains and the severity of the disease they produced in man, has been confirmed by most subsequent observers. However, in contrast to the above-noted experiences of Pfeiffer it is now generally accepted that, regardless of the character of the manifestations which they produce in man, the virulence of different cholera strains is apt to vary within fairly wide limits. Discussing this problem, Gotschlich & Weigang stated that the unequal virulence of the various strains might be due to differences in the rate of multiplication of the growths in question or to innate racial differences, and adduced some evidence suggesting that both these possibilities were of actual importance.

As shown by some early workers, such as Haffkine (1892) and Gotschlich & Weigang (1895), and confirmed by ample further observations, passage through intraperitoneally infected guinea-pigs is an effective means to restore or, if serially repeated, even to enhance the virulence of cholera strains. Gotschlich & Weigang referred in this connexion to one strain, the minimal lethal dose of which was reduced through 7 passages directly from animal to animal from a value of over 2600 million organisms to 900 million. Even more spectacular results were recorded by Kabeshima (1918a) in the case of an El Tor strain which had been subjected to passage through a series of 45 guinea-pigs.

Claims that the virulence of cholera strains could be enhanced by other means, e.g., through growth in diluted immune serum (Hamburger, 1903) through symbiosis with other bacteria (Puntoni, 1913a) or through short-term exposure to a temperature of 48°C (Sulman, 1923), seem not to have been supported by further observations. The same seems to hold true of the contention of Melnik (1925) that, as shown by guinea-pig experiments, (a) cholera vibrios which had been cultivated on agar became maximally virulent when 16 hours old but rapidly lost their virulence, whereas (b) growth of the organisms in broth led to a slow increase of the virulence (maximum after 4 days), followed by a gradual decrease.

Recently the problem of the virulence of V. cholerae has been studied once more by Jude & Gallot (1955—see also the preliminary communication of Gallot & Jude, 1954) as well as by Husain & Burrows (1956).

The French workers, intraperitoneally infecting lots of white mice with saline suspensions of one stock strain and four recently isolated and immediately lyophilized cholera strains, found that the virulence of Ogawa strains of V. cholerae for the white mouse varied according to the incubation temperature of the growths, those grown at 18°C proving most virulent,

¹It is also interesting to note that cholera strains which had become dependent for their growth on streptomycin were, according to Olitzki & Olitzki (1955), avirulent for mice when given intraperitoneally in mucin in doses up to 10⁹ organisms, and for guinea-pigs if suspended in broth in doses up to 4 x 10⁹ organisms.
and those incubated at 41.5°C proving least so. Incubation of the growths at 37°C led to a progressive loss of virulence, which became more marked as the number of subcultivations increased and which, like the virulence loss of the cultures grown at higher temperatures, was but partly reversible. Considering these findings and the absence of any differences when the sera raised against strains cultivated at these three temperatures were used for absorption and cross-agglutination tests, Jude & Gallut were led to conclude that

"The variations of the virulence under the influence of the temperature of incubation seems to be conditioned solely by variations in the toxigenic power [of the strains]."

[Trans.]

In the course of an exhaustive study on the virulence of cholera vibrios for the mouse, Husain & Burrows (1956) examined a total of 47 serial isolates of *V. cholerae*, obtained either from 15 patients who had recovered from the disease or—in four instances—from sufferers who had succumbed to it, in the sixth transplant from the original isolation plates by the conventional LD50 method. Results indicated that no clear correlation existed between the mouse virulence of the organisms, used in 5% mucin suspensions for intraperitoneal inoculation of the animals, and the virulence of human cholera, as judged by clinical criteria. On the contrary, it appeared that (a) "nonfatal cholera in man can be caused by vibrio strains of mouse virulence as great as that of strains from fatal cases, and also by strains of much less virulence for the mouse"; and (b) as far as the experiences of the authors went, fatal human cholera was not invariably due to an infection with strains of a high mouse virulence. Hence the conclusion reached was "that the mouse LD50 criterion of virulence is, in fact, quite irrelevant to cholera in man."

However, resorting to periodic quantitative examinations of the blood of the infected mice, Husain & Burrows were able to classify their strains as follows:

(1) Smooth strains of predominantly fatal origin, which increased rapidly in the blood, "possibly as a consequence of an increasing rate of dissemination from the peritoneal focus of infection, multiplication in the blood stream, or failure of defense mechanisms to remove them, or any combination of these factors". However, the maximal level of bacteraemia, reached just before deaths began to occur in the lots of mice concerned, remained moderate, possibly because of invasion of other tissues from the blood.

(2) Smooth strains of predominantly non-fatal origin increasing in the blood only at a moderate rate, "possibly because the spillover from the peritoneal cavity does not increase as the infection progresses or because multiplication in the blood is insignificant".

(3) R strains, which appeared to be limited in their ability to spread from the focus of infection in the peritoneal cavity, and reached only moderate numbers in the blood and then declined.

Discussing the significance of these and related findings, Husain & Burrows stated that
If fatality in the human infection may be taken as an indicator of the relative virulence of *V. cholerae* for man, the data reported here suggest that such virulent strains of the microorganism are demonstrably more invasive in the mouse. There is no a priori reason for such an association since Asiatic cholera is an extreme example of a true enteric infection, one in which there is little or no invasion of the tissues from the focus of infection in the lumen of the bowel. The greater invasiveness of vibrio strains of fatal origin is perhaps no more than an association with, or a reflection of, other elements of virulence of the microorganisms more nearly related to the pathogenesis of the disease in man.

Be this as it may, the studies of Husain & Burrows lend support to the postulation that, as far as cholera in man is concerned, the virulence of the infection is not merely a function of the toxicity of the causative organisms.

**Antigenic Structure**

*Early observations*

The scanty references made to the antigenic structure of *V. cholerae* during the period immediately following the introduction of the agglutination test as a means for the identification of this organism by Gruber & Durham (1896), are of historical interest rather than of actual importance. While, as summarized by Meinicke, Jaffe & Flemming (1906), Gruber & Durham as well as some other early observers assumed that differences in the virulence of the various cholera strains were the cause of their different agglutinability with a given immune serum, a few workers, such as Durham (1901) and Kolle & Gotschlich (1903), postulated that differences in the receptor apparatus of the organisms accounted for the discrepant serological results. This assumption was refuted by Meinicke and co-workers, who declared "that the cholera cultures possess the same receptors in about equal quantities, but that the avidity of the single receptors to the antibodies of the cholera serum differs in the various cultures". However, Kraus, Hammerschmidt & Zia (1911) re-asserted that cholera strains were apt to vary in their antigenic structure and that the presence of special agglutinogens was the cause of the discrepant behaviour shown by the strains which had been isolated during the 1908 outbreak in Kamaran, Arabia. Kraus and his co-workers insisted, however, that the different behaviour of these strains could be demonstrated only with immune sera possessing a low titre. Similarly, Ohta (1914) claimed that the action of low-titre sera was different from that of sera with a high titre and that with the aid of the former the cholera vibrios could be divided into two types.

Definite proof of the existence of different serological types of *V. cholerae* was adduced through a study of over 200 strains by Kabeshima (1913), to whose observations detailed reference will be made later in this chapter.

Greig (1916), testing 39 more or less haemolytic vibrio strains isolated from Calcutta surface waters, found "that the antigenic character of
vibrios isolated from water is different from that of the standard cholera vibrio as known to bacteriologists". As shown by cross-agglutination tests with sera obtained by immunizing rabbits with these water vibrios, 32 of them fell into 6 serological groups, while 7 remained ungrouped. Included among the latter were two strains of apparently atypically behaving cholera vibrios.

Observations by Mackie & Storer (1918), afterwards confirmed and amplified by Mackie (1922), showed that "par cholera" vibrios isolated in Egypt from patients with choleraic disease, were likewise serologically distinct from true cholera vibrios.

That the last-mentioned and also some earlier observers, even though they worked with agglutinating sera which were not fully specific according to modern standards, obtained surprisingly clear-cut results, was—as Gardner & Venkatraman (1935b) aptly pointed out—due to the fact that:

"The agglutination method used by Greig, Mackie, etc., involving a low temperature (37°C) and a relatively short period of incubation (2 hours), reveals in general only 0-agglutination, and so enables a distinction to be made between the various 0 subgroups."

The validity of this contention is proved by the conclusions of Mackie (1922) who maintained that:

"[a] By direct agglutination tests, using plain saline emulsions and incubating at 37°C for 2 hours, the par cholera vibrios are distinctly differentiated from V. cholerae;

"[b] V. cholerae antiserum exhibits apparent co-agglutination under certain conditions towards par cholerae A and certain similar types; this effect develops more slowly than the agglutination of the homologous organism and is of lesser degree and of lower end-titre; it is most markedly elicited when formol-broth emulsions are used and the tubes are incubated first at 55°C."

Heat-stable (O) and heat-labile (H) antigens

While the observations recorded above indicated that the cholera vibrio possessed an antigenic structure different from that of the cholera-like vibrios, and also that the latter fell into numerous immunological groups, they failed to explain the nature of these differences and—more generally speaking—furnished beyond some rather vague speculations no clue as to the character of the antigenic make-up of the organisms.

An investigation of the latter problem was rendered possible through the classical studies of Weil & Felix (1920) on the antigenic make-up of typhoid and paratyphoid bacilli, even though the two workers concluded from some preliminary tests that, in contrast to these bacterial species, the V. cholerae had no double antigenic structure. Observations suggesting that this organism possessed thermolabile as well as thermostable antigens were recorded in 1921 by Miyake and by Watanabe, but these findings, published in Japanese medical journals, attracted no attention. Brutsaert (1924) supported the tentative conclusion reached by Weil & Felix, stating that:
"Our anti-cholera sera obtained by injection of Koch's vibrios heated during 2 hours at 100°C, agglutinated our different strains of cholera vibrios, whether they were heated to 100°C or not, quite as well as anti-sera of 56°C prepared at the same time. As absorption tests confirmed the agglutination experiments, we state that all their antigen is thermostable." [Trans.]

Soon afterwards, however, the fine studies of Balteanu (1926) proved that the conclusion arrived at by Weil & Felix and by Brutsaert was due not to the absence of thermolabile antigens in the *V. cholerae* but merely to the difficulty of demonstrating their presence.

In the introduction to his paper, Balteanu stated that:

"The motile bacillus possesses two distinct kinds of agglutinable substances, one labile, the other stable when subjected to 100°C, or to dilute acid or absolute alcohol. The antisera contain special agglutinins corresponding to each kind of antigen. The labile factor and its agglutinins are responsible for agglutination in large, loose flocculi; the stable factor and its agglutinins for agglutination in small compact granules. The isolated flagella react as if composed entirely of labile material. The agglutinins for the labile and stable antigens are in the inverse order susceptible to heat: those for the labile antigens resist a temperature of 70°C for 20 minutes; those for the stable are inactive after such treatment. The non-motile races of normally flagellate organisms contain only the stable antigen and their properties are limited thereby; they agglutinate only in small granules and their agglutinins are destroyed at 70°C. This is the standard scheme of serological properties which has been kept in view in studying the antigenic complex of *V. cholerae*.

In his initial studies of this problem Balteanu used, besides the polyvalent immune serum of the Lister Institute, London, sera of his own, prepared by immunization of rabbits with suspensions of a cholera culture which had been heated before injection for 30 minutes at 58°C and for 2 hours at 100°C respectively. Carrying out agglutination and absorption tests with the aid of these sera, he was able to prove that the four cholera strains studied by him possessed H as well as O antigens. However, there was sometimes a poor contrast between the floccular (H) and the granular (O) forms of reaction—obviously due to the fact that in the case of the monoflagellate *V. cholerae* the ratio of heat-labile constituents was low as compared to that met with in the *Proteus* and paratyphoid groups. However, Balteanu was able to overcome this impasse by shaking and then centrifuging the suspensions of cholera agar cultures, so as first to liberate the flagella and then to separate them from the bodies of the vibrios, which were mostly thrown down during centrifugation. He found that the clear fluid obtained in this manner, in which only very few bodies of vibrios but abundant flagellar material were present,

"(1) agglutinates in typical flocculi with an ordinary anticholera serum made with an emulsion of whole cholera vibrios killed at 58°C.; it no longer functions after being heated at 100°C.;

"(2) When inoculated into rabbits it induces the production of a serum which makes flocculent clumps with a flagellar suspension and reacts almost exclusively with the flagellar labile constituents of an ordinary emulsion of the vibrios."
Balteanu's general conclusions, which have been confirmed by all subsequent observers, were that in *V. cholerae*:

"(1) There are two series of antigenic substances which may be termed stable (‘O’) and labile (‘H’) respectively (‘Somatic’ and ‘Flagellar’ antigens according to the terminology of Th. Smith). Of these the stable elements resist a temperature of 100°C for a considerable period while the labile elements are thereby destroyed.

"(2) The agglutination of the stable constituents by themselves (as illustrated by the agglutination of steamed suspensions) takes a purely granular form; the reaction of the isolated labile elements (as shown by the agglutination of living emulsions and flagellar suspensions by ordinary immune sera previously absorbed with steamed cultures) is definitely flocculent.

"(3) The combined reaction of the labile and stable constituents in living emulsions to immune sera made with ordinary cultures leads to a mixed flocculent and granular type of clumping except at the upper limit of the titre where the looser and more fluffy type is dominant."

It is of interest to add that the immotile opaque variant of *V. cholerae* met with by Balteanu (discussed in the section on “Cultural variation” in Chapter 3, page 126), behaved in the main like an “O” form, since no heat-labile “H”-agglutinable substances could be demonstrated, while the heat-stable, somatic, “O” factor was conspicuous. However, serum produced with this variant contained H agglutinins.

A further most important contribution to the knowledge on the antigenic structure of *V. cholerae* was made by Shousha (1931a, 1931b) through a study of two strains which had been isolated at the El Tor quarantine camp from pilgrims not suffering from choleraic disease and which were found to be agglutinable with one of the two available cholera-immune sera. Shousha was able to establish that the receptors which these two strains had in common with true cholera vibrios were heat-labile (flagellar) "group" receptors, whereas the somatic (O) receptors of the two suspect vibrios were quite different from the somatic antigen of *V. cholerae*. Shousha stressed, therefore, the importance of using in cholera laboratory work adequately heated suspensions of the cultures to be tested or of preparing sera by immunizing animals with heated suspensions of the organisms so as to produce sera free from H agglutinins. He also recommended with great reason that subcultures of the strains used for this purpose be issued together with the sera for the purpose of control tests.

Abdoosh (1932), examining 22 strains of true cholera vibrios, six strains labelled El Tor as well as three "paracholera" strains and 24 other cholera-like vibrios, found that none of the cholera-like vibrios possessed the same somatic antigen as *V. cholerae*. However, three of the strains labelled El Tor had the same thermostable and also the same thermolabile antigen as the classical non-haemolytic cholera vibrios, while one was agglutinated by cholera H->O serum, but not by cholera O serum. Abdoosh advised in this connexion that it was essential "to confine the term 'El Tor'
to the group of vibrios sharing with *V. cholerae* both its antigens, but differing in their being haemolytic.

Besides the so-called El Tor strain mentioned above, two of the cholera-like vibrios were agglutinated by H-O cholera serum in the living state but not after they had been heated for two hours at 100°C. Abdoosh concluded therefore that the heat-stable antigen of *V. cholerae* was "sharply specific" for this organism and the El Tor vibrios in the strict sense. However, some cholera-like vibrios, though differing in their somatic antigens, were related to the cholera vibrios by virtue of their heat-labile antigen.

Gohar (1932b), examining 45 cholera and cholera-like strains, maintained on the contrary that the relationship of the latter to *V. cholerae* "may refer to either the flagellar or somatic antigen". In his opinion, therefore, absorption tests were necessary in addition to agglutination tests in view of the fact that the cholera-like strains possessing the same antigens as *V. cholerae* were found incapable of absorbing all the antibodies from a cholera serum.

However, the importance of O-agglutination for the laboratory diagnosis of cholera was again emphasized by Taylor (1934) and White (1934a) in reports rendered to the Office International d'Hygiène Publique in response to an inquiry into the preparation of standard agglutinating sera for this diagnostic work. The observations recorded in this connexion by Taylor indicated that smooth cholera strains could be best distinguished with the aid of heated suspensions of the organisms, while White maintained that:

"The identification of *V. cholerae* depends in fact on the O agglutinins and it seems necessary to envisage the opportunity to make the diagnostic tests with a pure 'O' serum, i.e. with a serum raised against a vaccine heated to 100°C or naturally devoid of flagellar antigen."

The validity of this proposal was fully confirmed by Gardner & Venkatraman (1935b), whose publication may be considered the charter of the present knowledge on the antigenic structure of cholera and cholera-like vibrios.

Gardner & Venkatraman used for their comprehensive studies 101 cholera and cholera-like strains which according to their biochemical reactions could be divided into: (a) "typical" vibrios, i.e., those producing acid without gas in glucose, maltose, mannite and saccharose, giving the cholera-red reaction, and not fermenting dulcite; (b) "atypical" vibrios, found to be divergent in one or more of these characters, but showing a general similarity to the typical vibrios; and (c) "non-fermenting" vibrios, markedly different from the previous groups by failure to acidify any of the above-mentioned carbohydrates and also by an inability to produce the cholera-red reaction or to liquefy gelatin.

To test these strains serologically, Gardner & Venkatraman worked with H-O and O suspensions, and with H±O as well as with pure O sera.

Having established that, in contrast to what was the case in the *Salmonella* group, formal did not inhibit the O agglutinability of vibrio suspensions, Gardner & Venkatraman prepared their H-O suspensions by the addition of 0.2% formal and 0.2% chloroform to 24-hour-old veal-broth cultures (pH 8.0). O suspensions were obtained by placing
dense harvestings from 24-hour-old agar cultures in saline for two hours into boiling water. Such prolonged heating was found to be indispensable to destroy completely the antigenic action of the H component, but it was noted that a few minutes' exposure to 95°-100°C was sufficient to remove the H agglutinability of the suspensions. Alcohol treatment and growth of the vibrios on phenol agar were also tried to destroy the H component, but gave no satisfactory results.

H+O sera were prepared by immunizing rabbits with formalized unheated suspensions, pure O sera were manufactured with saline suspensions which, as noted above, had been exposed to boiling temperature for 2 hours.

Confirming and amplifying the observations of previous workers Gardner & Venkatraman were able to establish that:

(1) As shown by cross-agglutination tests with unheated suspensions and O sera, the "cholera group" of vibrios, i.e., the above-mentioned categories of "typical" and "atypical" vibrios, possessed a diversity of specific O antigens, six of which, being met with in more than one strain, rendered it possible to classify most, though not all, of the organisms of the group into six subgroups. All the standard stock strains of *V. cholerae* examined and also the majority of races isolated from patients with typical epidemic cholera fell into one group, called "I", and the same held true of the majority of the haemolytic vibrios tested, which were thus identified as El Tor vibrios in the strict sense.

(2) As demonstrated by the action of O sera on boiled suspensions, there existed in addition to the specific O antigens a common O antigen, the nature of which could not be definitely elucidated. The evidence regarding a possible extension of the non-specific O agglutination to vibrios outside the cholera group was also not conclusive, but Gardner & Venkatraman drew in this connexion attention to White's observation (1934b) on "Q" antigens which will be discussed later in this chapter.

(3) As shown by agglutination tests with formalized unheated broth suspensions and H-O sera, the vibrios of the cholera group possessed a common H antigen.

Gardner & Venkatraman urged on account of their experiences that for the identification of *V. cholerae* a standard subgroup "I O" serum should be used in conjunction with tests for haemolysis.

Before dealing with further investigations regarding the antigenic structure of *V. cholerae* in general, attention has to be devoted to the evidence on the existence of serological races of this organism as well as to the antigens present in dissociated vibrios.

**Serological races**

It is the merit of Japanese observers, and especially of Kabeshima (1913; see also Kabeshima, 1918b), to have definitely established the existence of serological races of *V. cholerae*. 
As summarized by Takano, Ohtsubo & Inouye (1926), Kabeshima based his observations on an examination of 195 cholera strains recently isolated in Japan and Formosa, and of 19 stock strains from European laboratories. He found that according to their serological reactions these strains could be divided into a "typical" and an "atypical" group, each of which agglutinated at high titre with homologous immune serum, but weakly with sera raised against strains of the opposite type. The presence of these two types could be confirmed with the aid of agglutinin absorption, complement-fixation and bactericidal tests. Kabeshima postulated that the different behaviour of the two types was due to the presence in each of two different antigens, a principal one, responsible for the reactions with homologous sera, and an accessory one, reacting with the heterologous sera. The strains isolated during the 1912 cholera epidemic in Japan were typical in character, while those derived in the same year from Formosa belonged to the atypical group.

The existence of two serological types of *V. cholerae* was soon confirmed by several other Japanese observers (see summaries by Nobechi, 1923 and Burrows et al., 1946). Pratt (1925) also described two serological types, the presence of which, though not invariably revealed by agglutination, could always be demonstrated by cross-absorption tests.

Nobechi (1923; see also Nobechi, 1933) proved the existence of a third serological type of *V. cholerae* standing between Kabeshima's two groups, which are now usually designated as the Inaba and Ogawa types. As summarized by Nobechi (1923), the strains of his new "middle" type (now often designated as the Hikojima type),

"are agglutinated by the sera of the two other types, capable of differentiating the strains of the two types from each other, to the same titre with corresponding strains; and the middle type sera, with no exception, agglutinate all strains of the other types as well as of the middle type almost uniformly high. From the result of the agglutinin absorption test, the middle type strains studied by the author are assumed to be provided with the common antigen X, and the original type specific A, at the same time also with the varied (i.e. "atypical") type specific B, though the development of the last is incomplete."

In his second paper (1933), Nobechi characterized the antigenic structure of the three types thus:

<table>
<thead>
<tr>
<th>Type</th>
<th>Specific fraction</th>
<th>Common fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original (Kabeshima's</td>
<td>A</td>
<td>X</td>
</tr>
<tr>
<td>&quot;typical&quot; group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate (Hikojima)</td>
<td>AB</td>
<td>X</td>
</tr>
<tr>
<td>Variant (Kabeshima's</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;atypical&quot; group</td>
<td>BC</td>
<td>X</td>
</tr>
</tbody>
</table>

Aoki & Oshiro (1934) claimed that the occurrence of specific thermostable antigens and/or of unspecific, partly thermolabile antigens accounted for the differentiation of *V. cholerae* into three types. According to this concept, the vibrios of the Inaba type had only specific receptors, those of the Ogawa group only unspecific receptors, while both kinds of antigen were present in the intermediate (Hikojima) type. However, as pointed out by Burrows et al. (1946), it is difficult to correlate this assumption with
what is known in regard to the H and O antigens of the cholera vibrio. In fact, the investigations of the workers quoted below leave no room for doubt that differences in the O receptor apparatus are solely responsible for the occurrence of the serological races of *V. cholerae*.

Scholtens (1933a; 1933b; 1934; 1936a, 1936b) stated in this connexion that the cholera vibrios fell immunologically into two groups, about two thirds of the strains possessing only an "A" antigen, the remainder also an additional antigen "B". Both these antigens, met with also in the El Tor vibrios, were found to be thermostable. Identical conclusions were reached by Heiberg (1936) as far as the *V. cholerae* was concerned.

Gardner & Venkatraman stated in a preliminary communication on their above-described investigations (1935a) that they had been able to confirm through agglutination and absorption tests the existence of "original" and "variant" types not only in cholera strains from Japan but also in races from India, China, and elsewhere as well as in El Tor strains in the strict sense. They added that:

"The reality of the third or 'middle' type is not yet fully confirmed, though some of our experiments indicate that certain of the Japanese races labelled 'middle' type possess, as they are supposed to, both the characteristic antigens of the original and variant types. Contrary to the belief of Inouye & Kakihara (1925), these characteristic antigens are of the heat-stable or O type. They are subsidiary or additional to the main heat-stable antigen that distinguishes them all from vibrios belonging to the other sub-divisions of the cholera group."

Whether these variants of *V. cholerae* were stable or fluctuating, was in the opinion of Gardner & Venkatraman still undecided. The claims made by some of the Japanese workers that they had succeeded in transmuting strains of the variant type into the middle type through growth in immune serum seemed not well substantiated, the less so because according to Gardner & Venkatraman (1935b) in some of the recorded instances at least such transitions had been concomitant with roughening.

In spite of these uncertainties there was, however, not the least doubt that at least two serologically distinct races of *V. cholerae* existed and Gardner & Venkatraman (1935b) urged, therefore, that the standard O sera used for cholera diagnosis should contain the subsidiary as well as the main agglutinins of the O subgroup I.

Further reference to the serological subgroups of *V. cholerae* will be made when dealing below with recent studies on the O antigens of this organism.

*R and p antigens*

That cholera vibrios which have undergone dissociation are apt to react peculiarly in serological tests seems to have been suggested first by attempts made by Hamburger (1903) to increase the virulence of these organisms: he noted in the course of this work that vibrios which had
been grown in specific immune serum (and which, as became later clear, had thus become rough) showed spontaneous agglutination when suspended in normal saline. This phenomenon was further studied by Kabeshima who, according to Takano and colleagues (1926), established already in 1913 that:

"When the cholera vibrio is cultivated in bouillon containing homologous serum, the organism becomes inagglutinable, but it acquires spontaneous agglutinability. This is due to the loss of specific receptors, and at the same time new receptors which are common to many strains are formed."

In a further publication (1918c), which was available to the present writer in the original, Kabeshima noted that spontaneous agglutination in 0.9% saline was shown not only by strains subcultivated repeatedly in broth containing homologous immune serum but also by 8 out of 19 old stock strains of *V. cholerae*. However, while these 8 strains remained capable of absorbing cholera agglutinins and also remained antigenic, those which had become spontaneously agglutinable in normal saline or inagglutinable with specific serum through growth in the latter, had lost their antigenicity as well as the property of agglutinin absorption—apparently because they had lost their specific receptors. Kabeshima also stated that the spontaneously agglutinating strains yielded homogenous suspensions if, instead of 0.9% saline, a 0.2% solution was used.

Shousha (1923) as well as Goyle & Gupta (1932), again studying the phenomenon of spontaneous agglutination of *V. cholerae* with a full knowledge of bacterial dissociation, confirmed the presence of profound differences shown by smooth and rough strains respectively in agglutination and agglutinin-absorption tests, but did not correlate these divergent reactions with changes in the receptor apparatus of the organisms. However, the latter problem received full attention in the studies of Yang & White and of White, which have been referred to in part in the preceding chapter.

As was noted there and as was also stated by White (1935a) in an article on "The serological grouping of rough vibrios", the serological specificity of the different vibrios depended on their smooth antigens. With roughening these differences tended to disappear so that forms which were quite distinct in the S state fell into larger R agglutination groups. Transition into the ρ form led even to the disappearance of this group specificity of the rough vibrios, so that, as White (1935) put it, "the serology of the ρ vibrio variant is overwhelmingly generalized."

In a further publication dealing with the ρ receptor complex of *V. cholerae* and its antibodies, White (1937c) reported on observations he had made when immunizing rabbits with polysaccharide fractions isolated from smooth cholera vibrios. These fractions were found to be actively antigenic, but the resulting sera showed a varying content of type- and group-specific agglutinins similar to that obtained in serum manufacture with whole vibrios. Besides being distinct by the range of their (type or
group) specificity, the receptor groups of the smooth vibrio polysaccharides were found to be partly alkali-labile and partly resistant to alkali. Immunizing a group of rabbits with a given polysaccharide, now one, then another of these various receptor groups was found to play a dominant role in the stimulation of antibodies. As pointed out by White, this uncertainty in agglutinin response was bound to complicate attempts to standardize cholera laboratory diagnosis by issuing standard antigens for serum manufacture in local laboratories.

Summing up further experiences regarding the rough and \( \rho \) antigens of *V. cholerae*, White (1940d) stated that

"it would seem that the major component in the somatic agglutination of \( R \) and \( \rho \) vibrios is a heat-stable antigen which, though it perhaps contains protein, is at least considerably resistant to proteolytic digestion. This component carries, with certain common receptors most obviously displayed in the reaction to \( \rho \) antiserum, the differential receptors of the variants and includes the polysaccharide \( C^\beta \) or \( C^\delta \). It is possibly to be regarded as the \( R \) or \( \rho \) antigen. But the somatic agglutinating apparatus of the variants seems to present other antigenic components, probably in the main common in quality to the \( R \) and \( \rho \) forms... Since they appear to be totally inactivated by proteolytic enzymes, they are probably of a protein nature. Possibly they are combined with the proteolysis-resistant component in a single complex."

In White's opinion, the \( R \) and \( \rho \) agglutinating antigens furnished the "skeletal system" of the cholera vibrio and, being less hydrophile than the smooth antigen, they indirectly conditioned the spontaneous agglutinability of the \( R \) and \( \rho \) variants by "failing to counteract the hydrophobe tendency of the surface lipoids".

"Rugose" antigen

Dealing with the immunological properties of the rugose variants of *V. cholerae*, White (1940a) stated that such races possessed a special antigen which proved to be resistant to heating in neutral solution at 100°C. Sera produced with this antigen reacted not only with the rugose variants of the \( O \) subgroup I of Gardner & Venkatraman, but also agglutinated rugose races of certain vibrios belonging to other subgroups. As White pointed out, the reactions obtained with such heterologous strains "disclose a flaw, actual if in practice unimportant, in the doctrine of the serological specificity of the heat-stable agglutinogens of \( O \) group I vibrios".

From rugose \( S \), \( R \), and \( \rho \) growths of cholera and El Tor vibrios a common, non-protein but carbohydrate-containing haptene could be isolated. This substance, which was found to be absent or inconspicuous in non-rugose strains, reacted strongly and characteristically with sera prepared from whole rugose vibrios.

Other additional somatic antigens

\( Q \) antigens. In a short preliminary note published in 1934, White stated that—notwithstanding the discrepant results recorded by some
earlier workers—there existed in the cholera vibrios an alcohol-soluble antigen comparable to the Q antigen previously isolated by him from salmonellae. The total Q fraction produced from agar-grown vibrios through alcohol extraction could be divided into (1) a soluble part (Q₁) which could be separated off by treatment of the total fraction with alkalized water, and (2) an insoluble Q₃ component which could be precipitated from the residue of the total fraction with the aid of hydrochloric acid.

Making further studies of these Q proteins of V. cholerae, White (1935b) was led to believe that the total Q fraction was identical with the "acid-soluble A substance" isolated, with the aid of extraction methods similar to those used by him, by Linton and his co-workers (see Linton & Mitra, 1934; Linton, Mitra & Seal, 1935; Linton, Shrivastava & Mitra, 1935).

The immunological properties of the Q antigens were characterized by White (1935b) thus:

"Vibrios heated at 100°C. in saline suspension agglutinate in a generalised manner and often to a high titre with the antisera of the Q proteins of the cholera vibrio. The antibodies concerned are not inactivated by the carbohydrate fraction of V. cholerae. Occasional strains of vibrio react similarly in the living state with these Q (cholera) agglutinins. The antiserum of the Q₂ substance of S (smooth) V. cholerae seems to possess agglutinating properties additional to those used by him, by Linton and his co-workers (see Linton & Mitra, 1934; Linton, Mitra & Seal, 1935; Linton, Shrivastava & Mitra, 1935).

Heat-labile somatic protein antigen (HLSP). As described by White (1940b), it was possible to extract from chloroform-treated young vibrio cultures with the aid of saline "a heat-coagulating antigen common to all known variant forms and seemingly derived from the deeper somatic substance". While, accordingly, this substance took no evident part in vibrio agglutination, it showed "extremely wide cross precipitation reactions throughout, but not overstepping, the vibrio group".

Heat-stable somatic protein antigen (HSSP). Using hot saline solutions for the extraction of chloroform-treated vibrio cultures, White (1940e) was also able to extract a heat-stable somatic protein antigen which, like the HLSP, appeared to belong to the deeply situated substances of the vibrios.

Anti-HSSP sera (prepared with the aid of rough and ρ strains to avoid an influence of the smooth antigen) gave intense precipitation reactions with extracts of R and ρ strains of cholera and many other vibrios and also with hot saline extracts of the smooth variants of the strains. However, the anti-HSSP sera "did not react visibly with any of the serologically active vibrio fractions... with the exception of Cγ (the polysaccharide fraction brought into solution on proteolytic digestion of R and ρ vibrios)" (White, 1936a).
Thus, as White (1940e) summarized:

“There have now been separated from the vibrio bodies (1) a heat-labile protein antigen (H.L.S.P.), (2) a heat-stable protein antigen (H.S.S.P.) possibly associated with a haptene Cy2, (3) an alcohol soluble ‘Q protein’ fraction . . .; and (4) the differential agglutinating S, R and ρ antigens with their respective polysaccharide haptens Ca, C9 and Cβ . . . Another haptene, Cy1, is probably also of somatic origin, while yet another, the rugose haptene . . ., has been derived from the intercellular secretion of rugose cultures. A method has been given for separating vibrio flagella. Antibodies for all these components occur or may occur in the sera of rabbits immunised with living cultures of V. cholerae. It is certain that vibrio cultures contain other separable serologically active and antigenic constituents and it is by no means unlikely that some of the fractions already described will prove to be mixtures.”

Recent observations on the O antigens

The necessity of using, according to the recommendation of Gardner & Venkatraman (1935b), O sera for the laboratory diagnosis of cholera was fully endorsed by large-scale investigations carried out in India and recorded by Taylor (1937, 1938, 1941).

Experiences identical with those in India were gained by Russo (1938), who recommended repeated (4-6) subcultivations of suitable cholera strains on agar containing 0.5% lithium chloride to obtain growths free from flagella for the preparation of pure O sera. Cultivation of the vibrios on alcohol-containing media or the use of heated suspensions were in Russo’s experience less suitable to obtain H-free antigens for serum manufacture, while growth of the organisms on phenol-containing media was altogether unsuitable for this purpose. He concluded from tests with 58 strains of cholera and cholera-like vibrios that with the aid of Inaba O serum it was possible to differentiate Inaba strains from Ogawa and El Tor strains as well as from the non-agglutinable vibrios of Finkler-Prior and Deneke.

An important study, based upon an examination of 50 cholera strains, 9 true El Tor strains and 11 strains of cholera-like vibrios falling into Gardner & Venkatraman’s O subgroups II-VI, was made by Burrows et al. (1946). They subjected, for this purpose, a group of representative cholera strains to a complete analysis of their heat-stable and heat-labile antigens by reciprocal absorption tests and studied at the same time the other vibrio strains with the aid of agglutination with monospecific immune sera. Verifying the tentative O antigenic formulae thus arrived at by absorption tests with known antigens, Burrows and his co-workers found the vibrio O antigens to consist of 13 components, five of which were considered as major antigens. One of the latter, designated A, was found only in vibrios of the O subgroup I and was, therefore, regarded as the group-specific antigen. Antigen B, found in 13 out of 20 Ogawa strains, but in no Inaba strain, and antigen C, met with in all of the 25 Inaba strains examined as well as in two Ogawa strains, were considered to be type-specific. The other major O antigens showed no association with the type-specific antigens or with one another. It thus appeared that four immunological types
existed within the O subgroup I, namely, type A, into which 11 of the 50 cholera strains tested fell, type AB, characteristic of the Ogawa strains, type AC, to which the Inaba vibrios belonged, and finally type ABC, inferred to correspond to the Hikojima type.

Since with the exception of the group-specific antigen A, which was met with exclusively in \textit{V. cholerae} and the El Tor strains in the strict sense, the major antigens were also found in vibrios not belonging to O subgroup I, Burrows and his co-workers urged that the identification of vibrios falling into this class "should be based on agglutination with monospecific A anti-serum".

Findings confirming those of Burrows et al. were recorded by Gallut (1949a, 1949b), who examined

(a) 49 authentic cholera strains, including 35 isolated during the 1947 epidemic in Egypt;

(b) 12 El Tor strains in the strict sense, partly those obtained during the 1938 Celebes outbreak;

(c) 21 cholera-like strains, 13 of which were of human origin and 8 isolated from water.

As summarized by Gallut, the percentage incidence of the 13 antigenic factors in cholera and true El Tor vibrios on the one hand, and in the cholera-like vibrios examined by him on the other was as follows:

<table>
<thead>
<tr>
<th>Antigenic factors</th>
<th>Cholera vibrios</th>
<th>Cholera-like vibrios</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>32</td>
<td>47</td>
</tr>
<tr>
<td>C</td>
<td>95</td>
<td>14</td>
</tr>
<tr>
<td>D</td>
<td>45</td>
<td>19</td>
</tr>
<tr>
<td>E</td>
<td>47</td>
<td>9</td>
</tr>
<tr>
<td>F</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>G &amp; J</td>
<td>1.6</td>
<td>19</td>
</tr>
<tr>
<td>H &amp; M</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>I &amp; K</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>L</td>
<td>47</td>
<td>28</td>
</tr>
</tbody>
</table>

Considering that, in contrast to the factor A, which had been met with only in true cholera and El Tor vibrios, the factors B and C, responsible respectively for the type-specificity of Ogawa and Inaba vibrios and jointly for that of the Hikojima type, were also present in the cholera-like vibrios, Gallut urged the use of monospecific anti-O sera A for the laboratory diagnosis of cholera. He also recommended that, in order to avoid co-agglutinations due to the presence of factors D or E, sera specific for the factors B and C be used for type differentiation. In regard to the selection of suitable strains for vaccine manufacture he maintained that:

"It is true that the solution so far adopted of preparing vaccine from strains isolated during the epidemic against which control measures are being taken is generally satisfactory, but the objection to it is that it is entirely lacking in precision. If the overriding
The necessity of having a completely polyvalent vaccine in stock seems acceptable, that is, a vaccine comprising the 13 O factors, it would, however, seem logical to take into account the antigenic composition of the vibrios responsible either for endemic cases or a specified epidemic. Only the complete analysis of a sufficient number of strains can furnish this indispensable information. [Trans.]

The validity of Gallut’s recommendations for vaccine manufacture was denied by Sokhey & Habbu (1950c) who (a) compared the mouse-protective power of vaccines prepared with some of Gallut’s strains possessing in part a complicated antigenic structure with that of two vaccines manufactured from Haffkine Institute strains, which had a quite simple antigenic structure, and (b) correlated these findings with determinations of the virulence of the strains in question for white mice. The conclusion reached was that the protective power of a cholera vaccine depended not upon the complexity of the antigenic structure of the strains used for their manufacture but upon the virulence of the strains chosen. Sokhey & Habbu suggested in this connexion that the complexity of the antigenic structure observed by Gallut might be due to the degeneration of the strains from age, because they were found to have no virulence to white mice.

Kauffmann (1950) and Singh & Ahuja (1950), approaching the problem with the aid of serological methods, were also unable to confirm the results of Burrows and of Gallut.

The main conclusion reached by Kauffmann, after an examination of 41 strains sent to him by Gallut as well as of six additional cholera strains was that “the occurrence of new types or variants within the O group I that were claimed to be characterized by the antigens D, E, F, G, H, I, J, K, L, and M could not be demonstrated.” However, while noting this statement, the present writer finds it impossible to share Kauffmann’s opinion that “technical errors in the planning and estimation of the serologic examination” accounted for the apparent occurrence of these antigens.

Kauffmann considered a polyvalent O serum, prepared with the aid of Inaba as well as Ogawa strains, to be the most suitable for the identification of V. cholerae and recommended for the differentiation of the two types of strains a serum obtained by absorption of a polyvalent or an Ogawa serum by an Inaba strain. He also advocated the manufacture of polyvalent cholera vaccines with the aid of Inaba and Ogawa strains without giving attention to the serological subtypes as Gallut had urged.

Singh & Ahuja (1950) thus summarized the experiences gained through a serological and biochemical investigation of (a) 96 cholera, El Tor, and cholera-like strains of their own, and (b) 49 strains put at their disposal by Burrows and by Gallut:

“Were we sceptical of the claim of Burrows et al. (loc. cit.) that a new type of cholera vibrio has been discovered, namely one containing cholera group-specific antigen only.
PROBLEMS IN IMMUNOLOGY

This has not been confirmed by using the type representative material supplied to us by Burrows and by Gallut.

"Of several thousand strains of V. cholerae tested by us with cholera 'O' serum (containing group-specific plus type-specific agglutinins) not one strain has yet been encountered which subsequently did not agglutinate with type-specific serum, either Ogawa or Inaba. Type 'A' serum supplied by Gallut has been found by us to be a non-differential serum containing cholera group-specific 'A' plus type-specific 'C' agglutinins. It is the same type of diagnostic reagent as is normally used for the preliminary identification of V. cholerae and is in no way superior in its diagnostic properties to the non-differential serum used at present in India.

"Antisera raised against so-called 'A' type vibrios—Burrows and Gallut types—were tested against strains of cholera vibrios including freshly isolated and old laboratory cultures. Not a single strain showed positive agglutination. In the light of our experience in India we are of opinion that the existence of a new type of cholera vibrio—containing antigen 'A' only—has not been established, nor have we been able to confirm the presence of cholera type-specific 'B' and 'C' antigens in non-cholera vibrios."

In agreement with these observations, Venkarraman (1953) recorded in the 1952 report of the Indian Council of Medical Research that an examination of 84 cholera strains, including 49 of the Ogawa type and 31 of the Inaba type besides 4 which had become rough, failed to show any culture possessing only the group-specific O antigen. He added that one of the rabbits which had been immunized with an Ogawa strain yielded a serum containing only type-specific Ogawa O agglutinin, being thus completely deficient in the group-specific factor.

In contrast to the above-mentioned observations, Wahba (1951), re-examining the 1947 Egyptian strains formerly tested by Gallut (1949) but excluding those which showed abnormal features (i.e., loss of agglutinability, spontaneous agglutination, positive results with Millon's reagent, or thermostability), confirmed the multiplicity of the antigenic factors demonstrated in V. cholerae by Burrows et al. and by Gallut. It is noteworthy, however, that Wahba found the antigenic formulae of the strains examined by him "not completely stable". He stated in particular that (a) the C factor was apt to disappear rapidly in aging cultures and was not demonstrable in formalized suspensions; (b) the factors D and E, which had been found to be absent in a number of the strains in 1948, were now present, while the L factor, previously demonstrated in several of the strains, had become absent. It was also noted that, while the results of agglutination tests became manifest after four hours as far as the major antigenic factors A-E were concerned, agglutination of the minor factors took place more slowly, becoming manifest only on the following morning.

Evaluating the results obtained by Wahba, it must be kept in mind that he worked exclusively with old strains. Thus, as pointed out with great reason by the reviewer of Wahba's article in the Tropical Diseases Bulletin, his paper "does not appear to help in clearing up the point at issue, which could best be settled by the examination of freshly isolated strains of V. cholerae."
Recent observations on the serological races of *V. cholerae*

As noted before, Burrows et al. (1946), agreeing with the views, but changing the symbols adopted by White (1937c), stated that the antigens B and C of the cholera vibrio were type-specific, while the antigen A was group-specific. The antigenic structure of the three serological types of *V. cholerae* was, therefore, as follows:

<table>
<thead>
<tr>
<th>Type</th>
<th>Antigenic structure according to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>White</td>
</tr>
<tr>
<td></td>
<td>Burrows et al.</td>
</tr>
<tr>
<td>Inaba</td>
<td>A X</td>
</tr>
<tr>
<td>Hikojima</td>
<td>A B X</td>
</tr>
<tr>
<td>Ogawa</td>
<td>B X</td>
</tr>
</tbody>
</table>

The classification proposed by Burrows et al. was also adopted by Gallut (1949a, 1949b) and by Kauffmann (1950), to whose postulations reference will be made below.

Kabeshima (1918b) had claimed that he had succeeded in transmuting his "variant" (Ogawa) strain into the "original" (Inaba) type by cultivation in homologous serum and also by inoculation into the gall-bladder of rabbits. Whether he also observed transmutations in the reverse direction seems uncertain. In analogy with Kabeshima's experiences, Nobechi (1923) was able to transmute through cultivation in homologous serum two "variant" strains into the "middle" (Hikojima) type. It is of great interest that observations recorded by Shrivastava & White in 1947 lent support to these early statements of Kabeshima and Nobechi.

Shrivastava & White recorded that they had been able

(a) to obtain in the case of 10 cholera and 3 El Tor strains of the Ogawa type through cultivation in Ogawa monospecific serum races which were indistinguishable from Inaba type strains; and

(b) to change, with the aid of monospecific Inaba serum, 4 out of 8 cholera strains which, though predominantly Inaba-like in serological reactions, possessed also an Ogawa factor, into the Ogawa type.

It proved impossible, however, to produce serological changes other than roughening in 5 cholera and 3 El Tor strains of the strict Inaba type through cultivation in homologous monospecific serum.

Discussing the significance of these observations, Shrivastava & White tentatively postulated:

"That the Ogawa serological complex represents the known acme of elaboration of the specific somatic antigen of *V. cholerae*.

"That this antigen is subject to degradation presumably by failure of the organism to synthesize certain chemical groupings.

"That this change is expressed serologically as a positive modification in the detail of antigen and not merely as a factorial loss.

"That from this debased Inaba antigen there is no easy return to the Ogawa state by a revival of lost synthetic power, the only escape from the interference of specific antibodies being in the 'rough' change, i.e. entire failure to synthesize the specific complex with resultant unmasking of the 'R' antigen."
It is interesting to add that disagreement with the postulations just quoted has quite recently been expressed by Bhaskaran & Gorrill (1957), who insisted that

"experiments with antisera carried out in the cold confirmed that growth of the culture subsequent to the addition of specific antiserum was not necessary for the isolation of the Inaba mutants from Ogawa cultures."

This observation, the two authors continued, made it very probable

"that these mutants were already present in the parent culture, and agglutination by antiserum only facilitated isolation of the mutant in the supernatant fluid. The rate of appearance of these mutants was of the order of 1 in $10^5$ cell divisions. The inability to demonstrate directly the presence of the mutants in Ogawa cultures was probably due to this low mutation rate, which would require the examination of a similarly large number of colonies for a chance isolation of the mutant... The failure to isolate Ogawa mutants from Inaba cultures with the aid of selective antiserum may have been the result of still lower reverse mutation rates."

However, Bhaskaran & Gorrill admitted that they had been unable to obtain "unequivocal evidence for antigenic type variation in Vibrio cholerae as a result of mutation."

The views on the antigenic structure of the serological types of V. cholerae expressed by Joya (1950) do not seem significant, because in the opinion of this worker thermolabile antigens played a more important role in the serological differentiation of Inaba and Hikojima strains than the thermostable antigens. This is not in accord with the generally accepted doctrine.

In the opinion of Kauffmann (1950), Hikojima and Ogawa strains, because they proved identical in cross-absorption tests, had to be considered one common type. He proposed, therefore, that a distinction should be made merely between two types of V. cholerae, namely the Inaba type (A C) and the Ogawa-Hikojima type with the antigenic formula A B (C). Kauffmann maintained in this connexion that the C antigen, though present in small amounts in the Ogawa vibrios, was not well developed in strains grown at 37°C, so that these were incapable of completely absorbing Inaba sera. However, if the strains were grown at 20°C, they completely or almost completely absorbed Inaba sera. In Kauffmann's opinion this was the case because at 20°C the B antigen of the Ogawa strains developed less abundantly and was thus incapable of inhibiting the development or "disponibility" of the C antigen.

It is important to note that Gallut (1953b), studying two cholera and two El Tor strains with the aid of single-cell cultivation, came to the conclusion that the Hikojima type, even though it showed sometimes a tendency to change into the Ogawa type, was a valid race of V. cholerae. Since Gallut was unable to find C antigen in Ogawa strains even if these had been cultivated at 20°C, he rejected Kauffmann's proposal to classify these and the Hikojima strains in a common group, as the latter worker had proposed.
As shown by these researches, the problem of the somatic antigens of *V. cholerae* is still far from being fully solved. It is, therefore, not surprising to find that within recent years the optimistic attitude adopted in this respect immediately after the publication of Gardner & Venkatraman’s basic observations has been replaced not rarely by one of doubt. Thus, as stated by White in a report rendered in 1948, many experienced cholera workers in India “for one reason or another cling to the view that vibrios other than *V. cholerae* may from time to time contribute to cholera”.

The validity of this view, which implies that the system of cholera laboratory diagnosis adopted on the basis of Gardner & Venkatraman’s findings on the somatic antigens of *V. cholerae* is not sufficiently comprehensive, was thoroughly discussed during joint meetings of the Cholera Advisory Committee of the Indian Council of Medical Research and the WHO Expert Committee on Cholera (1952), held at New Delhi in 1951. Though the speaker introducing this subject went so far as to consider Gardner & Venkatraman’s characterization of the *V. cholerae* merely “a preconceived notion”, in the opinion of the WHO experts, “the gaps still existing in the knowledge on this subject did not detract from the practical value of the tests adopted for the laboratory diagnosis of the infection. Endorsing this opinion, the committee reached the conclusion that the present definition of the cholera vibrio, though incomplete, was sufficient for practical purposes.” [Page 6].

Acceptable though this conclusion remains, there is a most vital need for further research on the *V. cholerae* O antigen. This ought to include (1) studies on the antigenic stability of the organism under varied conditions of culture and storage; (2) further inquiry into the O antigen complex, especially as regards concealed and blocking antigens; and (3) the application of both old and new information to the study of the O antigen complex of vibrio strains of precisely known history, and with a minimal number of transplants intervening between isolation and study.

**Recent experiences on the H antigens**

Vassiliadis (1936a) found that treatment of cholera-vibrio suspensions with chloroform (1.5 ml per 10 ml of suspension), while leading to a considerable reduction of the O-agglutinability of the organisms, markedly increased their H-agglutinability. More than that, some strains of cholera-like vibrios, which were not agglutinable with *H--O* cholera-immune serum in their original state, showed considerable H-agglutinability after they had been treated with chloroform. As stated by Vassiliadis in a second paper (1936b), this difference was probably due to a removal of lipoids inhibiting H-agglutination by the chloroform. The reduction of O-agglutinability through the action of this reagent was presumably due to the dissolution of lipoids necessary to bring about agglutination.
A method for isolating the flagellar fraction of vibrios was described by White (1940c), who recommended using for this purpose R or ζ cultures on account of their freedom from the smooth specific antigen.

The procedure was started by adding chloroform to a dense saline suspension of the vibrios and then stirring so as to separate the flagella from the bacterial bodies. The extract obtained by centrifugation of the chloroform-treated growths after dispersal in saline was treated with an equal volume of saturated ammonium-sulfate solution. After 24 hours’ contact with the precipitant the flagella could be collected and washed with the aid of brisk centrifugation.

The preparations obtained in this manner proved suitable in dilution for H-agglutination tests, while dense suspensions could be used for the absorption of flagellar agglutinins. Injection of the preparations into rabbits stimulated the production of apparently pure flagellar agglutinins.

Following up the work of Gardner & Venkatraman (1935), who had shown that vibrios possessing different specific O antigens had the same H antigen, Taylor, Pandit & Read (1937) tested 558 strains of cholera-like vibrios with different sera, including an Inaba H – O serum and a serum manufactured with a chemically not fully defined but mainly protein-containing extract of Inaba vibrios. The interesting fact was established that, while these two sera agglutinated, besides V. cholerae strains, also many of the cholera-like strains, this held true only of those giving a cholera-red reaction.

Ahuja & Singh (1939), making further studies on 219 vibrio strains which were not agglutinated with specific cholera O serum (subgroup I of Gardner & Venkatraman), found that 35.5% of these cholera-like strains possessed H antigens partially or completely identical with the H antigen of V. cholerae. As shown by cross-absorption tests carried out with 10 of these strains and pure H sera (prepared by absorption of sera raised against living suspensions of these strains with massive doses of heat-killed cultures of the homologous vibrios), the cholera-like vibrios agglutinable with cholera H + O serum apparently fell into three groups, namely,

1) those possessing an H antigen identical with that of V. cholerae;
2) strains, the major portion of whose H antigen was identical with that of the cholera vibrios; and
3) strains possessing besides a major individual H fraction a minor one identical with the H antigen of V. cholerae.

The cholera-like strains which were inagglutinable with cholera H + O serum, possessed mainly individual H antigens, though some showed a partial H relationship among themselves.

A detailed analysis of the H antigens of V. cholerae was made by Burrows et al. (1946) through reciprocal absorption tests carried out on a representative group of 10 cholera strains with the aid of H + O sera which had been absorbed with their homologous O antigens. The H antigenic structure
of these strains was found to be of a complexity similar to that of the O antigens, but only ten components of the H antigen could be detected, one of which was common to all of the strains. Apparently no correlation existed between the variant distribution of the individual H and O antigens respectively.

A further study, carried out with agglutination tests only on a larger group of vibrios, including, besides cholera and El Tor strains, strains of cholera-like organisms, indicated "an apparently random distribution of the H antigens in both cholera and non-cholera vibrios".

Kauffmann (1950) stated that he had been unable to confirm the existence of subtypes of the H antigen.

_Specially prepared antigens_

Basu, Chaudhury & Basu (1940) stated that they had obtained a thermostable antigen by (a) immersing a Cellophane or collodion bag filled with sterile normal saline in a growing culture of _V. cholerae_ in peptone solution, and (b) filtering the contents of the bag after five days' incubation through a Chamberland L, candle.

The diffusate obtained in this manner contained carbohydrate substances but practically no protein. Injected into rabbits, it gave rise to specific agglutinins and precipitins and also protected these animals against lethal doses of _V. cholerae_.

According to a report published in 1947, Feigina, Kuzin & Shapiro obtained through trypic digestion of cholera vibrios an antigenic complex which, however, was far less active than the glucido-lipoid antigen of Boivin & Mesrobeanu. Injected into rabbits, the trypic digest stimulated the appearance of agglutinins but not of precipitins. Hydrolysis of the digest, the antigenicity of which seemed to be due to the presence of peptides, led to the separation of nitrogen-containing substances and the loss of antigenic power.

_Chemical constitution of the antigens_

An early attempt to extract the antigens of _V. cholerae_ with the aid of alcohol was made by Levaditi & Mutermich (1908). The residue of their extracts, obtained through centrifugation and evaporation of the supernatant, proved to be antigenic both in complement-fixation tests and in rabbit experiments, and conferred active immunity to guinea-pigs. It was apparently thermostable.

Since the validity of these findings was doubted by Prausnitz (1911), a further and thorough study of this matter was made by Landsteiner & Levine (1926, 1927).

These two workers obtained by extraction of saline-washed cholera vibrios with hot 75% alcohol and further extraction with ether and hot absolute alcohol a solution,
the sediment of which, separated off after cooling (a) reacted in high dilution (1/500,000) in precipitin tests with cholera-immune serum, and (b) acted as an antigen when injected into rabbits.

While this crude substance gave both protein and carbohydrate reactions, the white powder obtained through purification with alcohol and other reagents was almost protein-free and no more antigenic, but continued to give precipitin reactions up to the above-mentioned titre and positive carbohydrate tests.

In Landsteiner & Levine’s opinion, these findings were compatible with the assumption that the crude extracts contained an antigenic complex consisting of protein and a specifically precipitable but non-immunizing complex carbohydrate substance, which probably belonged to the class of “residue” (residual) antigens.

Investigations to demonstrate the presence of such residual antigens (haptens) in cholera-like as well as in cholera vibrios were made by Jermoljewa & Bujanowskaya (1930). They extracted for this purpose the washings of 24-hour-old agar cultures, after digestion with caustic potash, with acetic acid and alcohol. The substances thus obtained were protein-free but gave reactions proving the presence of carbohydrates. The extracts gave precipitin reactions with cholera-immune serum but were not antigenic when injected into rabbits, unless gelatin or normal pig-serum had been administered simultaneously.

Linton (1932) extracted a carbohydrate-like fraction from cholera and cholera-like vibrios with the aid of the following technique:

“The organisms were sown on Roux bottles and incubated for 48 hours. The growth was then washed off in normal saline, and the solution brought to an acidity of N/20 with glacial acetic acid. . . . The bacterial mass was boiled on a sandbath under a reflux condenser until coagulation occurred. The coagulated mass was allowed to cool . . . and then run several times through a Sharples supercentrifuge until a semi-opaque brownish solution was obtained. This solution was precipitated with three volumes of 90% alcohol and placed in the icebox overnight. The heavy precipitate was pipetted off, separated from the alcohol as completely as possible by centrifuging, and taken up in 200 or 300 cc. of water. Insoluble matter was discarded and the solution again precipitated with alcohol. As before, the precipitate was freed from any remaining insoluble matter, and dissolved in 100 cc. of water, where it formed a clear, brown-tinted solution, with a faint but unmistakable biuret reaction. It was strongly acidified with glacial acetic acid, and boiled. After cooling, the dark brown flocculum which had appeared was centrifuged off, and the supernatant fluid, which was now biuret negative, was precipitated with three volumes of alcohol. The precipitate was dried, weighed and dissolved in approximately N/20 NaOH to make a 1% solution.”

As Linton added, the solutions thus obtained, while giving negative biuret, Millon’s, and xanthoproteic reactions, proved positive with Molisch’s reagent even at extremely high dilution and, after boiling with dilute acids, were found capable of reducing Fehling’s reagent. In cross-precipitation tests the carbohydrate fractions obtained with the aid of the method described above from cholera-like as well as from cholera vibrios gave positive results not only with their homologous immune sera but with all
sera tested which comprised, besides five raised against *V. cholerae*, one manufactured with a water vibrio. Thus, as Linton put it, the carbohydrate fractions, “if not identical, are at least closely related in the agglutinating and non-agglutinating vibrios” (i.e., in cholera and cholera-like vibrios). The carbohydrate fractions obtained in an identical manner from typhoid and dysentery (Flexner) strains failed to react with any of the six above-mentioned sera.

The results of further studies on the immunochemistry of the vibrio group by Linton and his co-workers, which have already received preliminary attention in the preceding chapter, will be dealt with later on.

As noted before, Boivin and his collaborators obtained with the aid of trichloracetic acid, from cholera vibrios as well as from other species of Gram-negative bacteria, extracts stated by these workers to represent the “total antigens” of the organisms in question. According to Boivin & Mesrobeanu (1935), the substances in question corresponded chemically to a complex combination of specific polysaccharides with fatty acids. Exposure of the complete antigens to heating in a weakly acid medium led to the separation of an insoluble portion containing the fatty acids from the polysaccharides, which remained in solution. The latter, which represented the residual antigens of the organisms, could be solidified by precipitation with alcohol or acetone. If redissolved, the residual antigens produced solutions which, in contrast to those made from the total antigens, were non-opalescent and weakly dialysable as well as non-antigenic.

On account of the rather fragile nature of the complete antigens, it was possible to split off the specific polysaccharides without trichloracetic extraction directly from the intact organisms by the use of “brutal” methods, such as heating of the bacteria in an acid medium. The same result could be produced by the action of the “diastases” of the organisms. Damboviceanu & Barber (1940), carrying out chemical analyses of the trichloracetic acid extracts of five cholera strains, confirmed that the complete antigen of *V. cholerae* was a glucido-lipoid complex which contained amino-nitrogen and phosphorus. The extracts did not give a biuret reaction and also failed to reduce Fehling's solution, but gave a feebly positive Molisch reaction.

Reviewing the experiences of Boivin and his co-workers as well as of subsequent observers in regard to the trichloracetic acid extraction of *V. cholerae*, Burrows et al. (1946) insisted that none of these workers “demonstrated either the biochemical homogeneity of these preparations or their postulated identity with the O antigen of the vibrios by cross absorption experiments, nor have the preparations been subjected to immunological analysis”.

Burrows and co-workers also laid stress upon the fact that the purified substances obtained by Burrows (1944) with the aid of organic solvents, which were found to consist of phospholipid and additional nitrogenous material, gave a negative Molisch reaction.
It has to be added that Linton et al. (1938) recorded that they had separated from a cholera strain, which had been isolated in the early stage of an outbreak, a glucolipid fraction. They deduced from this observation that the presence of such a complex might be characteristic of an epidemic type of the organism. Apparently, however, no further observations confirming this assumption have been made.

Attention has been drawn in the third chapter to the observations of Linton and his co-workers (see Linton, 1940, 1942), who were able

(a) by racemization with dilute alkali solutions to demonstrate the presence of two types of protein 1 in the vibrios, the first of which was usually present in V. cholerae; and

(b) also to show the existence of three types of vibrio polysaccharides, most cholera strains being found to possess those of type I (galactose and an aldobionic acid consisting of galactose and glycuronic acid), but a considerable minority showing type II polysaccharides, in which arabinose instead of galactose was found to be combined with an aldobionic acid of the same composition as in the type I polysaccharides.

As maintained by Burrows et al. (1946) and also admitted by Shrivastava, one of Linton’s principal co-workers, in a 1951 summary, the relation of these polysaccharide types to the H and O antigenic structure of the cholera vibrios and to their differentiation in serological races is not clear. It is important to note in this connexion that, as summarized by Shrivastava (1951), the polysaccharide fractions initially isolated by Linton and his colleagues were found to be serologically inactive and that those prepared later by Shrivastava & Seal (1937) and by Linton, Shrivastava & Seal (1938) and Linton et al. (1938), though giving precipitin reactions with suitable sera raised against intact vibrios, were found to possess no antigenic properties, thus falling into the category of haptens.

As will be gathered from statements made above, in the course of his immunological investigations White was also able to make observations on the chemical character of the various vibrio antigens demonstrated by him. The following supplementary statements have to be made in this connexion.

(1) Non-protein carbohydrate-containing specific substances

As stated by White (1936b; see also 1936a),

"The protein-free polysaccharide specific substance was prepared in a suitable manner by digesting vibrios washed in alcohol and boiled in saline with 1% papain at pH 5.5 for 7 to 8 hours at 90°C, centrifuging the mixture and precipitating the active substance from the supernatant with alcohol, extracting the active material from this precipitate with a saturated aqueous solution of picric acid and then reprecipitating it with alcohol. Picric acid was removed by reprecipitation with alcohol."

As mentioned already, White (1937c) found these preparations actively antigenic.

1 These proteins have been further studied by Mira, (1933) who, though finding marked differences in the respective structure of their molecules, admitted that it is impossible with the present data to say whether proteins I and II represent two different entities or whether they are mixtures of several proteins".
(2) **Q antigens**

Describing the chemical properties of the protein Q antigens, White (1934b) stated that both the Q₁ and the Q₂ antigens gave a positive biuret test, the former antigen reacting less intensely than the Q₂. Both precipitated with Millon's solution, the colour of the precipitate turning to pink or red at room temperature. In contrast to Q₂, the Q₁ antigen was soluble in dilute hydrochloric acid. Both antigens proved to be readily soluble in alcohol in the presence of HCl. The solvent action of acetic acid was considerably less marked.

(3) **Other special antigens**

Basic chemical reactions shown by the other special vibrio antigens which White (1940b, 1940c, 1940e) described, may be tabulated thus:

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Biuret test</th>
<th>Millon test</th>
<th>Molisch test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-labile somatic</td>
<td>intensely positive</td>
<td>intensely positive</td>
<td>definitely positive</td>
</tr>
<tr>
<td>protein antigen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat-stable somatic</td>
<td>strongly positive</td>
<td>strongly positive</td>
<td>strongly positive</td>
</tr>
<tr>
<td>protein antigen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flagellar antigen</td>
<td>intensely positive</td>
<td>imperfect</td>
<td>weakly positive</td>
</tr>
</tbody>
</table>

*Note:* The rugose hapten isolated by White (1940a) gave a negative biuret reaction but an intensely positive Molisch reaction.

Important as these and the previously discussed related observations of White are, they merely characterize the chemical composition of the vibrio antigens in a general manner. In fact, as stated by White (1937c), it was impossible to decide whether his specific carbohydrate-containing antigen was strictly a polysaccharide or a polysaccharide-containing complex of the type described by Boivin and collaborators and also by other workers.

Shrivastava, Singh & Ahuja (1948) tried, for further studies on the immunoochemistry of *V. cholerae*, in addition to the above-described method of White (1936b) the following two methods:

(a) "The centrifugate of the growth for 72 hours in papain-digested mutton broth is concentrated *in vacuo* at a temperature of 40°C. to 45°C. and the concentrate worked for the isolation of polysaccharides [Shrivastava & Seal, 1937]. Protein is removed from the precipitate by shaking it with chloroform and butyl and amyl alcohol."

(b) "Phenol method [Palmer & Gerlough, 1940], in which the acetone-dried growth of the bacteria is treated with 90 per cent phenol. This dissolves away the protein and liberates the polysaccharides."

The final product obtained in bulk from an Inaba strain of *V. cholerae* with the aid of the last-mentioned method, which was found most suitable, proved soluble in distilled water and 0.85% saline, biuret-negative, and Molisch-positive in a dilution of 1:100,000. The nitrogen and acetyl group contents were 7.7% and 2.1% respectively. The substance reacted
up to a titre of 1:200,000 with immune sera prepared against both Inaba and Ogawa subtypes of *V. cholerae* and, administered subcutaneously to white mice in two doses of 0.2 ml each at weekly intervals, conferred to the animals thus treated a high degree of immunity against intraperitoneal infection with mucinized suspensions of *V. cholerae* (Inaba subtype).

Singh et al. reported in 1950 upon further studies of the *V. cholerae* polysaccharides, isolated through phenol treatment of acetone-dried growths and subsequent precipitation with 95% alcohol. It was possible to obtain with the aid of this method fractions of high antigenicity from Ogawa as well as from Inaba subtype strains. As was established in the course of this work, polysaccharide fractions which precipitated at high titre with cholera-immune serum did not necessarily confer a high degree of protection to mice. Another interesting finding was that intravenous injection of a polysaccharide complex isolated from an Inaba strain into guinea-pigs which had been passively immunized with monospecific or non-differential cholera-immune serum, did not produce signs of anaphylaxis.

In contrast to the observations recorded above, Sato et al. (1950) found that the polysaccharide fractions isolated by them from Inaba and Ogawa strains of *V. cholerae* were non-antigenic. Possibly, however, this was the result of de-acetylation, due to the alkali treatment which had been used for extraction.

Purified, polysaccharide-free protein fractions, which were also tested, took, in the opinion of Sato et al., "a prominent part in the type-specific antigenicity in complement fixation tests and agglutinin absorption tests". These are rather surprising results, needing confirmation.

Krejci, Sweeney & Jennings (1949) reported that they had separated with the aid of electrophoresis from Inaba and Ogawa cholera strains three main constituents, of which two ("X" and "B") showed antigenic activity. It appeared that the heat-labile antigens were associated with the X constituents, the heat-stable O subgroup I antigen with the B constituents. These were found to contain polysaccharides in combination with proteins. The X constituents appeared to consist mainly of proteins or of lipids.

A further exhaustive study of the fractions of *V. cholerae* separable with the aid of electrophoresis has been made by Burrows (1957), who used for this purpose 32 of the strains previously examined by Husain & Burrows (1956) with the results summarized above. The technique used by Burrows was briefly as follows:

Suspensions of the strains, which had been grown for 18 hours on thionine-glycerol agar, were filtered through gauze and then heated in the water-bath at boiling temperature for 1 hour. The heat-stable bacterial material thus obtained was next treated for 40 minutes in a Mickle apparatus with about 1/4 volume of glass beads so as to ensure as completely as possible a rupture of the cell walls and a liberation of the soluble contents. After the supernatant had been decanted, the beads were washed three times with distilled water and the resulting fluids were pooled with the supernatant obtained first. The pooled material was filtered through paper, centrifuged at high speed and the yellowish,
slightly opalescent supernatant, which represented the intracellular substance of the organisms, was decanted and dried from the frozen state. This material was found to contain about 30% protein, 15% polysaccharide and 15% ash.

The cell wall substance, which had been separated from the intracellular material in the manner described above, was washed four times in distilled water, then resuspended and treated in an oscillator for 1 hour. Afterwards the resulting highly turbid suspension was clarified by high-speed centrifugation, and the supernatant was decanted and dried from the frozen state to be available for testing. It was found that this material contained about 30% protein and 60% polysaccharide, but only negligible amounts of ash.

The main results of a study of the electrophoretic mobility of the protein and polysaccharide components of both the cell wall and intracellular fractions obtained were that

"O strains of fatal origin were characterized by the presence of relatively larger amounts of faster moving intracellular polysaccharide and cell wall protein "

and that

"The relative proportions of some of the mobility fractions changed in an apparently systematic way in serial isolates and this was taken to represent an in vivo variation in the microorganisms."

Hence, as Burrows put it, *V. cholerae* appeared to be "heterogeneous within the limits of its accepted characterization ". It will be important to see how far these changes are correlated to differences in the virulence and toxicity of the organisms.

**Serological Reactions**

*Early observations*

Profound studies on cholera immunity led Pfeiffer (1895a) to the recommendation of a serological method for the differential identification of *V. cholerae*. He thus described the technique of this test which is now known under the name of "Pfeiffer's reaction":

"As a rule I use cholera serum, the titre \( \frac{1}{10} \) of which is at least 0.001, and take of it for each test 0.01, i.e., ten times the minimal effective dose. One loop of the culture to be tested is mixed with 1 ml broth and the above-mentioned serum dose, and injected intraperitoneally into young guinea-pigs of 200-300 g... The syringe used for this purpose is provided with a blunt canula. The resistant corium is split with the aid of scissors and the blunt end of the canula penetrates thus quite easily into the peritoneal cavity. After 20 minutes I remove with the aid of glass capillaries droplets of the peritoneal contents for examination in hanging drop and stained preparations. If after that time still numerous well-preserved and motile vibrios are present in the peritoneal cavity, the reaction is negative and cholera bacteria are, therefore, absent. If on the contrary after 20 minutes in the exudate the injected comma bacilli are found to be changed into granula, among which only quite few and immotile vibrios are noted, there are two

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1 Pfeiffer (1895) designated as titre of a cholera-immune serum " that minimal quantity of serum which, if injected together with the cholera dose in 1 ml broth into the peritoneum of young guinea-pigs of 200 g, just suffices to lyse 2 mg of the normal virus within 1 hour ."

As "normal virus " he designated cholera cultures possessing a virulence sufficient to kill guinea-pigs within 24 hours, if injected intraperitoneally in doses of 1/5-1/10 loop (0.4-0.2 mg) of a 20-hour-old agar culture.
possibilities: (1) the test culture is devoid of pathogenic properties and thus rapidly destroyed even in normal animals, or (2) true cholera vibrios are present, which are lysed by the specific bactericidal substances (positive reaction).” [Trans.]

To decide this issue, a control guinea-pig was used, which received intraperitoneally 1 loop of the culture to be tested + 0.01 ml normal serum in 1 ml of broth. If droplets of this mixture removed from the peritoneal cavity after 20 minutes showed the presence of viable organisms, the diagnosis of cholera was confirmed. If, on the contrary, the organisms in the exudate of the control animal had disappeared, the result of the test was doubtful in so far as avirulent cholera vibrios as well as cholera-like vibrios were apt to be affected by the normal serum. Pfeiffer recommended testing under these circumstances the antigenicity of the culture in question through immunization of guinea-pigs and stated that it had been possible to confirm in this indirect manner the true nature of an old avirulent Calcutta strain of V. cholerae. Pfeiffer admitted that thus the results of his test were bound to be more reliable the higher the virulence of the cultures under examination was, but added that “as a rule it is absolutely impossible not to come to a decision”. In his opinion, an application of the above-described test was indicated only in the case of atypically-behaving strains from stools and of water vibrios, whereas the hitherto adopted methods sufficed to arrive at a reliable diagnosis in most instances.

As had been noted already, Gruber & Durham introduced in 1896 the expedient and therefore generally applicable method of agglutination for the laboratory diagnosis of cholera. Achard & Bensaude claimed in the following year that advantage could also be taken of this method by testing the sera of cholera patients with known cholera cultures.

Kraus (1897) noted that, as had been previously shown in the case of the typhoid bacillus, cholera vibrios remained agglutinable with specific serum after they had been killed by heating at 56°C. He also made the important observation that the addition of specific immune sera to germ-free filtrates of cholera broth cultures led to the formation of precipitates. As proved by controls with normal sera as well as with sera raised against other bacteria, this was, like agglutination, a strictly specific test. Since identical reactions could be produced with the juice obtained by exposure of a mixture of cholera vibrios and glass dust to a pressure of 300 atmospheres, the precipitinogens appeared to form part of the bodies of the organisms instead of being excreted by them.

1 Bordet had already noted in 1895 that, in contrast to normal rabbit serum, the sera of rabbits immunized against cholera first immobilized and then promptly agglutinated V. cholerae and that, unlike the bactericidal power, this property was not lost when the sera were heated to 55-60°C. Nevertheless, as summarized by Fitzgerald & Fraser (1928), Gruber & Durham “for the first time described the agglutination reaction as a separate and distinct characteristic of immune sera”. This statement is also valid as far as the observations recorded in 1896 by Pfeiffer & Vagodes are concerned, because these two workers considered the phenomenon of agglutination of cholera vibrios observed by them to be due to the causes producing Pfeiffer's reaction and, in contrast to Gruber & Durham, referred merely in a tentative manner to the practical value of agglutination tests.
Though, as summarized by Hetsch (1912, 1928), the question of the interrelations existing between the above-described antibodies as well as the problem of their immunological importance soon became the subject of considerable dissensions. Early practical experiences, particularly the large-scale investigations of Kolle & Gotschlich (1903), endorsed the specificity and consequently the diagnostic importance of the reactions concerned, particularly of the agglutination test.

A somewhat divergent opinion was expressed by Friedberger & Luerssen (1905), who claimed that the usefulness of the latter method was limited in that in the case of freshly-isolated strains, after an incubation at 37°C for 6-8 hours, the vibrios showed spontaneous agglutination ("pseudo-agglutination") in normal saline. Since, however, in actual practice agglutination tests are made after longer intervals (at an average after about 18 hours) when, as admitted by Friedberger & Luerssen, spontaneous agglutination had become absent, their observations would be of little importance even if generally valid. However, as shown by subsequent investigations, especially by the experiences of Kabeshima (1918c), who examined 160 freshly-isolated cholera strains in this respect with negative results, the phenomenon of pseudo-agglutination, observed by Friedberger & Luerssen in the case of only 11 strains, must be rare.

As already alluded to in Chapter 3, the discovery of the El Tor vibrios (Gotschlich 1905, 1906) which, though giving serological reactions identical with those of the classical V. cholerae, appeared to be different from the latter on account of their apathogenicity and their haemolytic properties, led to most serious dissensions in regard to the specificity of the serological tests. Ruffer (1907), one of the protagonists of the school claiming a separate status for the El Tor vibrio, went so far as to conclude

"That it is not advisable to trust to the agglutination test only in the bacteriological diagnosis of cholera. The test is useful but not specific."

The German workers, on the other hand (see summary by Kolle & Schürmann, 1912), who denied the existence of qualitative differences between the cholera and El Tor vibrios, continued to maintain that

"the system of cholera diagnosis, which is based largely upon the immunological reactions, still rests upon a fully secure scientific foundation and has as well proved its practical value". [Trans.]

Though it remains legitimate to evaluate the differences existing between the classical cholera vibrio and the V. El Tor either in favour of the unity of the two or to support the concept of their separate standing, modern investigations have left no room for doubt that, as far as their basic serological reactions are concerned, the two organisms do not differ. Evidence in this respect, additional to that furnished in the preceding section of the present disquisition, will be brought forward in the following pages.
Further investigations on Pfeiffer's reaction and bacteriolysis

As shown by investigations of Baumgarten (1921), it is possible to use mice in place of guinea-pigs when performing Pfeiffer's test. However, as noted by Hetsch (1928), the results obtained with the aid of this modification were not as uniform as those with Pfeiffer's original technique, presumably because complement, which is indispensable for bringing about the reaction, is not as plentiful in mice as in guinea-pigs.

According to the summary of Hetsch (1928) the outstanding value of Pfeiffer's test carried out in the classical manner was confirmed by numerous observers. He noted in this connexion that, while cholera-immune sera failed to react with cholera-like vibrios, the latter reacted typically with sera manufactured with homologous or serologically identical organisms. *Vice versa*, the immune sera raised in this manner gave negative tests with cholera vibrios.

Harvey (1929), though admitting that Pfeiffer's reaction had been largely superseded by agglutination tests, upheld its value "in difficult sporadic cases of cholera-like disease". In view of the availability of the highly specific O sera it is a moot point whether this statement is still valid at present.

In the course of his investigations, Pfeiffer devoted attention to the question of to what extent his reaction could be produced outside the living organism.

Pfeiffer (1894b) established in this connexion that, if a broth suspension of cholera vibrios mixed with a dose of potent immune serum was injected into the peritoneal cavity of freshly-killed guinea-pigs and the carcases were kept in the incubator, marked bacteriolysis took place for the first 20 minutes but did not progress further.

As Pfeiffer observed in 1895, dilutions of cholera-immune serum in broth, which proved highly vibriolytic when injected into the peritoneal cavity of guinea-pigs and the carcases were kept in the incubator, exerted *in vitro* no bactericidal action on cholera vibrios and even formed a suitable substrate for their multiplication. If, however, a 1% dilution of cholera-immune serum in broth was injected intraperitoneally and droplets of the peritoneal contents of such animals removed after 20 minutes were seeded with cholera vibrios, bacteriolysis took place but often did not become complete.\(^1\)

*In vitro* bacteriolytic tests, to be used side by side with, or in place of, Pfeiffer's reaction, have been recommended by several authors.

Thus Serekowski (1906) mixed various saline dilutions of the sera to be tested with constant quantities of cholera vibrios and of complement (normal serum) and, after incubation at 37°C for 4-6 hours, used the mixed material for pouring agar plates, which were kept in the incubator for 24 hours. The absence of growth, or the number of colonies which had by then developed, indicated to what extent the serum dilutions in question possessed bacteriolytic properties.

Using a similar technique, Amako (1909) added to two parts of the various serum dilutions to be tested 1 part of a suspension of cholera vibrios and 1 part of normal rabbit

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\(^1\) This method was also used by Bordet (1895). Craster (1914), giving priority to the latter worker, stated that he had utilized "Bordet's test" to demonstrate bacteriolysis *in vitro* side by side with Pfeiffer's reaction for a study of cholera-like vibrios.
serum diluted 1/10. The tubes were kept at 37°C for 1 hour, then smears were made and stained with dilute carbol fuchsin in order to determine to what degree bacteriolysis had taken place. At the same time amounts of 0.01 ml from each tube were used to pour gelatin-agar plates. Colonial counts were made after an incubation at 37°C for 24 hours.

Prausnitz & Hille (1924), besides confirming the above-mentioned observations of Pfeiffer, also found it possible to reproduce the phenomenon of bacteriolysis in vitro with the aid of adequately-graduated amounts of immune serum and complement, particularly if fresh complement was added from time to time. Bacteriolysis became still more marked if also limited amounts of an exudate, which had been obtained through intraperitoneal injection of a guinea-pig with sterile broth, were added to the tubes.

An in vitro test for bacteriolysis with the aid of peptone water has been described by Kiribayashi (1931b). As summarized in the Tropical Diseases Bulletin (1932), his technique was as follows:

"A loopful of a 20-hour agar culture of test vibrio is suspended in peptone water (peptone 3; sodium chloride 5; dist. water 1,000) of pH 7.6 which is isotonic with the serum components of the test. A comparison is made by setting up two sets of dilution mixtures, the one containing inactivated immune serum, complement and suspension of test organism and the other, a control, containing inactivated rabbit serum, complement and suspension. Specific bacteriolysis is indicated after 3-5 hours by the absence of turbidity in the first set of mixture and positive turbidity in the control set."

Gordon & Johnstone (1942) stated that with the aid of bactericidal tests with normal guinea-pig serum it had been possible to detect antigenic differences between true cholera strains and cholera-like vibrios and even to single out cholera strains which differed antigenetically from the main group (see also preliminary observations made in the latter respect by Mackie & Finkelstein, 1931). In the opinion of Gordon & Johnstone, the bactericidal technique "may be of use in differentiating between strains of V. cholerae for which the agglutination technique is not practicable, and as an alternative method to the haemolytic test for distinguishing between strains of V. El Tor and true cholera strains."

Ahuja (1951) and Singh & Ahuja (1951), noting that fresh guinea-pig serum exerted a marked vibriocidal effect on rough or partially rough cholera vibrios, while leaving smooth vibrios unaffected, recommended the following test for the detection of roughness in V. cholerae strains:

An 18-hour-old peptone water culture of the strain to be tested was diluted 100-fold with the same medium. One part of this dilution was mixed with two parts of 1 in 2 complement (diluent peptone water). The mixture was incubated for 4 hours at 37°C. The initial inoculum and the 4 hours' growth in the presence of complement were then sampled by plating 3-mm loops on agar plates without spreading, and results were read after incubation at 37°C for 18 hours.

It was found that rough or partly rough cholera vibrios either failed to grow or grew to a greatly reduced degree in the presence of complement, an effect which was inhibited by heating the mixtures at 56°C. Since 20% of the guinea-pig sera tested did not exert this effect, it was essential to make preliminary tests with known smooth strains.

While Gallut (1953a) did not consider the above-described test fully reliable, Dudani (1955), after an examination of 51 cholera growths, reassessed the value of the method of Singh & Ahuja.
It may be conveniently added that, as recorded by Popescu (1924),
the washed blood-platelets of cholera-immunized rabbits produced not
only agglutination but also lysis of cholera vibrios. It is also of interest
that, according to the experiences of Pacheco & Peres (1940), mucin reduced
or even inhibited the vibriolytic action of cholera-immune sera (inactivated
by heating at 55°-56°C) in the presence of complement. The differences
between the lytic action of cholera-immune sera and that produced by
bacteriophage were studied by MacNeal, Frisbee & Krumwiede (1937).
In contrast to the former, bacteriophage lysis was transmissible in series
and rapidly led to variations in the size and form of the vibrios.

General agreement exists that bacteriolysins are present in the sera of
normal persons at rather low titres only. Papamarku (1917) noted in this
connexion that while some previous observers met usually with titres
varying from 0.1 to 0.75 in the sera of their controls, he found that 3 out of
16 such normal individuals had bactericidal titres of 0.05, while the others
had titres below this figure.

As summarized by Svenson (1909), the presence of bacteriolysins in the
sera of cholera patients or convalescents was demonstrated by several
early workers, such as Lazarus (1892), Metchnikoff (1893), Pfeiffer (1894a),
and Amako (1909), who as a rule resorted to animal experiments, especially
Pfeiffer’s test, but occasionally relied only upon in vitro tests. As found by
these and other observers, the bactericidal titre of such sera (i.e., the
minimal amount still protecting guinea-pigs against intraperitoneal cholera
infection) varied considerably and was, according to Metchnikoff, even
in the case of past severe infection sometimes not higher than that of normal
sera. In the experience of this worker, during the acute phase of the disease
bactericidal substances were present in but small amounts in the sera of
not more than 45% of the sufferers. However, as established by him and
all other observers, the bacteriolytic titre rose during convalescence, to
become maximal usually during the second and fourth week. In the ex­
perience of some but not of all workers quoted by Svenson, the protective
value of the convalescent sera was not higher than that of normal sera after
6 weeks.

Svenson himself was able to demonstrate the presence of bacteriolysins
in 89% of the 27 convalescents examined by him within the second to
fourth week after onset of the disease. There was no parallelism between
the presence of bacteriolysins and that of agglutinins, found in the sera of
only about one-third of these persons. In view of the fact that Pfeiffer’s test
was negative in the case of some convalescents who had survived severe
cholera attacks, Svenson concluded that the appearance of bacteriolysins,
because frequent, was a characteristic sign of recovery, but did not fully
account for it ( ... eine Begleiterscheinung, die sehr häufig bei der Genesung
beobachtet wird und ein charakteristisches Symptom derselben ist, mit
derselben aber nicht unbedingt identifiziert werden darf).
Referring to observations made in regard to the persistence of bacteriolysins in the sera of persons who had recovered from cholera, Papamarkou (1917) stressed that obviously these immune bodies were apt to become inconspicuous or even absent at a time when the individuals in question were still fully immune against the disease. A case in point was that of Pfeiffer, whose serum showed no specific immunizing properties only three months after he had been affected by cholera.

Further noteworthy observations on the presence of bacteriolysins in the sera of cholera patients or convalescents may thus be summarized:

Shiiba & Oyama (1920) demonstrated the presence of bacteriolysins in the sera of 97 convalescents with the aid of the Neisser and Wechsberg test. As a rule, though not invariably, the results thus obtained ran parallel with those of agglutination tests.

Tagami & Watanabe (1920), also applying Neisser and Wechsberg’s method, obtained positive results in 89% of the 91 sera tested. Bacteriolytic tests were found to yield results earlier than agglutination tests, becoming positive in 80% to 85% of the cases in 1 to 3 weeks. The bacteriolytic titres decreased after the third week, only half of the sera still proving positive after one month. In the experience of these two workers, “the agglutination and bactericidal reactions do not run parallel, but sometimes quite oppositely”.

Ukil (1928), studying 30 convalescent sera collected from cholera patients in Calcutta, 25 of which agglutinated the causative organisms at titres ranging from 1:100 to 1:1000, found that 18 of these sera possessed marked bacteriolytic properties, while seven produced less marked, and five weak reactions in tests made in vitro.

Continuing such tests (platings from tubes containing a mixture of two drops of the convalescent serum under test, 4 drops of a suspension of V. cholerae containing 2000 million organisms per ml, 2 drops of 50% complement and 0.6 ml normal saline, which had been incubated at 37°C for 4 hours), Ukil & Guha Thakurta (1930) found that the bacteriolytic properties of the convalescent sera increased progressively, reaching their maximum at the time when the faeces of the individuals tested no longer yielded positive cultures, i.e., usually 1-3 weeks after onset of the disease.

As far as it was possible to continue the observations, it appeared that the bacteriolytic properties of the convalescent sera remained manifest for several weeks. The presence of bacteriolysins was confirmed by rabbit experiments, a dose of 0.5-1 ml intravenously as a rule protecting the animals against intravenous injection of a lethal dose of V. cholerae. It was found that 85% of the convalescent sera tested agglutinated cholera vibrios at titres ranging from 1:100 to 1:2000.

Attempts to demonstrate the presence of specific bacteriolysins in the sera of cholera patients with the aid of a modified Pfeiffer test gave, according to a statement made in the 1941 report of the Indian Research Fund Association, no fully satisfactory results. Though sometimes present early in the disease, bacteriolysins could be demonstrated in but 33% of the sera examined. In a majority, the bacteriolysins showed no type specificity.

Observations on the presence of bacteriolysins in the sera of healthy cholera carriers are not numerous. Massaglia (1911) claimed that bacteriolysins as well as agglutinins were present in the sera of such individuals in the same amounts as in the sera of convalescents, and postulated, therefore, that the freedom of the carriers from manifest signs of cholera was due to the presence of immune bodies in their blood. De Bonis (1912), since he was unable to demonstrate bacteriolysins
or agglutinins) in the sera of healthy carriers even within the days immediately following the isolation of *V. cholerae* from their faeces, reached on the contrary the conclusion that the freedom of the carriers from clinical manifestations of the infection could not be the result of a general immunity but must have depended upon other factors, possibly a local immunity.

Levi della Vida (1913), though able to demonstrate the presence of agglutinins in all but 9 of the 48 convalescent and healthy carriers examined by him, found bacteriolysins less regularly demonstrable, so that they were absent from some of the well-agglutinating samples. On the other hand, bacteriolysins were never detected in sera devoid of agglutinating power.

Sano (1921) found like de Bonis that the sera of healthy cholera carriers contained practically no immune bodies, while Toguchi (1919), as quoted by Takano, Ohtsubo & Inouye (1926), recorded that “the immunological reactions of the carriers are not uniform, and in some cases they are not stronger than those of the healthy person”. In accord with de Bonis, Toguchi assumed that the absence of clinical manifestations of cholera in the carriers must “be explained on some basis other than the immunological reactions of the blood serum”. A similar opinion was expressed by Satake (1926) who was able to demonstrate the presence of bacteriolysins in but one out of 5 cholera carriers and apparently found agglutinins in none of them.

Classical investigations by Kolle (1896, 1897) showed that administration of killed cholera vaccines in a single high dose (1/10th of a culture) to 17 individuals led to a most marked increase of the originally almost invariably low bacteriolytic titre of their sera. As far as could be established, the increase of the titres became manifest already 6-10 days after vaccination and was still demonstrable for periods up to about 12 months (350 days) after the vaccine had been administered.

While several of the subsequent observers maintained in agreement with Kolle that a rise of the bactericidal titres did not become demonstrable before the fifth day after vaccination, some noted an earlier increase of the bacteriolysins. Balteano & Lupu (1914), examining two individuals vaccinated for the first time with doses of 1 ml, spoke in this connexion of the fourth day after administration of the prophylactic. Aaser (1910) drew attention to three persons in whom a considerable rise of the bacteriolytic titres (twice to 0.01, once even to 0.003) had been noted already on the third day after vaccination. Ahuja & Singh (1948), examining 21 persons once inoculated with 1 ml of cholera vaccine, likewise established that the sera of these individuals showed vibriocidal properties after three days.

It is unanimously stated that the initial appearance of bacteriolysins in the sera of cholera-vaccinated persons is followed by a further increase of the bacteriolytic titres. However, the statements as to when their maxima
are reached and as to how long the titres remain at this level vary consider-
ably. Thus according to Ahuja & Singh (1948) the maximum of the bac-
teriolytic titres was attained about the 8th day after vaccination and a fall
of the titres became manifest by the 30th day. Sano (1921) noted that the
bactericidal titre in the sera of cholera-vaccinated persons reached an
acme in three weeks, while Balteano & Lupu (1914) found that in two
persons vaccinated against cholera for the first time the bacteriolytic titre
became maximal not sooner than after 56 days.

Though it is generally agreed that the drop of the bactericidal titres
setting in after their maximum had been reached and persisted for some
time is gradual, observations regarding the length of the period during which
bacteriolysins remain demonstrable in the sera of cholera-vaccinated
individuals at increased titres gave divergent results. As noted already,
Kolle found in some of the persons vaccinated by him conspicuous bac-
tericidal titres even after a period of about 12 months. Sano (1921) noted
that these titres fell as low as they were in normal blood-sera within
10 months. Hetsch (1928), summarizing the observations made in this
respect during the First World War maintained that as a rule the increase of
the bacteriolytic titres persisted not longer than 7-8 months. In the ex-
perience of Ahuja & Singh (1948), the level of the bacteriolysins in the sera
of cholera-vaccinated individuals was by the 100th day already but slightly
higher than before immunization. Papamarku (1917), examining 60 persons
at varying times after cholera vaccination, noted that Pfeiffer tests, made
with the sera collected from 31 of these individuals between the 11th and
134th day after immunization were positive in 61%, whereas an identical
result could be obtained in only 8% of 28 persons tested during a period
of from 146 days to about 300 days after vaccination.

The few observations made in regard to changes in the bactericidal
titres as a result of revaccinations against cholera gave strikingly divergent
results. Balteano & Lupu (1914), while maintaining that in the two persons
who had received only a single dose of cholera vaccine, a temporary drop
of the immune bodies, including the bacteriolysins, was noticeable, stated
that in those persons who had received one or two further doses at weekly
intervals, no such "negative phase" was present, the bacteriolytic titres
rising rapidly and reaching a maximum of 1:150, which was maintained
up to the end of the observation period of three months. Karwatzki (1906b)
found that the bactericidal power of the sera of 11 individuals, which did
not greatly increase after the first administration of cholera vaccine, was
most markedly enhanced after a second dose had been given five days later.
Ahuja & Singh (1948) stated on the contrary that injection of a booster
dose six months after the initial cholera vaccination did "not increase the
vibriocidal power of the serum to any marked extent compared to the
effect produced by the primary stimulus". Aaser (1910) even noted in the
case of one person who had been revaccinated with 2 ml of cholera vaccine
20 days after administration of an equally high initial dose, a rapidly setting in, but temporary drop of the bactericidal power. It is of great interest that Papamarkou (1917), revaccinating guinea-pigs three weeks after an initial administration of cholera vaccine, also noted such a drop of bactericidal power, but established that nevertheless most of the animals in question resisted during the persistence of this “negative phase” intraperitoneal challenge with lethal doses of *V. cholerae*.

**Haemolysins**

Supplementing the information already furnished in the third chapter, it has first to be noted that, though giving consistently negative results in standard haemolytic tests, according to some workers the classical cholera vibrios were able to lyse goat and sheep red blood corpuscles under peculiar conditions. The following observations have to be recorded in this connexion:

Doorenbos (1932) claimed that it was possible to transform through bacteriophage action non-haemolytic cholera vibrios into haemolytic vibrios which thus showed the properties of *V. El Tor*. Having also found that 24-hour-old cultures of non-haemolytic cholera vibrios possessed anti-haemolytic properties, being capable of inhibiting haemolysis of sheep erythrocytes through *El Tor* vibrios, Doorenbos reached the conclusion that “the presence or absence of haemolytic properties depends solely upon the proportion of haemolytic and anti-haemolytic elements present in the strain in question”.

Reporting in a later article upon the examination of 12 cholera strains which had been isolated one to two months previously at Calcutta from the dead bodies of cholera victims and had been forwarded to Alexandria, Doorenbos (1936a) stated that all these strains were incapable of producing haemolysis of sheep erythrocytes after cultivation for 24 hours, but that after incubation for only 8 hours, 4 of the strains exerted a marked and 5 a feeble haemolytic action.

Doorenbos (1936b) further maintained that, in analogy with the findings made in the case of *V. cholerae*, young (6-8 hours old) *El Tor* cultures showed more marked haemolytic properties than those grown for 24 hours. He also claimed the existence of strains which, on account of their feeble haemolytic properties, stood half-way between the classical *V. cholerae* and the true *El Tor* vibrios.

Vassiliadis (1935b) stated that cholera vibrios which had been cultivated in broth containing 5 per 1000 glucose, showed in contrast to those grown in ordinary broth haemolytic properties for sheep erythrocytes and also recorded (1935a) that two originally non-haemolytic cholera strains produced marked haemolysis after they had been passed three times through ordinary broth and subcultivated for a fourth time in glucose broth. Vassiliadis further stated (1935b) that he had been able to produce through immunization of rabbits not only with *El Tor* vibrios but also when using instead a non-haemolytic cholera strain for this purpose, anti-haemolysins inhibiting the haemolytic properties of filtrates from *El Tor* cultures. He also claimed that the cholera-immune serum routinely used in his laboratory for agglutination tests neutralized the *El Tor* haemolysins at the same titre as anti-haemolytic *El Tor* sera. Vassiliadis concluded from these observations that a non-active haemolytic antigen was present in the classical *V. cholerae*.

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1The occurrence of a “haemolytic phase”, becoming manifest in otherwise typical cholera strain, during the sixth and twelfth hours of growth on agar, has been recorded again by Fournier (1940).
It is noteworthy, however, that in the experience of Goyle (1939) immune sera raised against typical non-haemolytic *V. cholerae* exerted no neutralizing action on the haemolysins of El Tor or other haemolytic vibrio strains.

Van Loghem (1925), studying 14 cholera and 4 El Tor strains, reached the conclusion that a haemolysin was produced by the latter organisms which reacted like an *exotoxin*: it was soon demonstrable in culture filtrates, was thermolabile and, injected into rabbits, produced an anti-haemolysin, as previously shown by Kraus & Pribram (1906). The haemolysin of *V. cholerae* appeared much later in the cultures and had the character of an *endotoxin*, being neither thermolabile nor antigenic. It was possible to demonstrate the presence of such an *endotoxin* also in old El Tor cultures.

Van Loghem recorded in a further communication (1926) that bacteriophage action hastened the liberation of the cholera endohaemolysin: whereas in filtrates of cultures, which had been acted upon by bacteriophage, marked haemolysis was demonstrable in 5-6 days, in the filtrates of cultures subject to autolysis only this phenomenon became apparent not sooner than after 8 or 9 days.

The observations of van Loghem were confirmed and amplified through interesting studies of Bernard, Guillerm & Gallut (1939a, 1939b, 1939c).

Bernard and colleagues (1939a) were able to extract with the aid of ammonium sulfate from 3-day-old El Tor agar cultures and even from 24-hour-old broth cultures or saline suspensions a substance which, redissolved in normal saline, proved strongly haemolytic for sheep erythrocytes. It was not possible to extract such a haemolysin from cholera cultures, but a feebly haemolytic substance could be extracted from saline suspensions or broth cultures of *V. cholerae* which had shown evidence of haemolysis after incubation for 5 and 9 days respectively.

In their second note (1939b) Bernard and co-workers recorded that addition of suitable quantities of El Tor or cholera vibrios to a 2.5% suspension of sheep blood corpuscles led to the production of a violet colour. Due apparently to a reduction, this phenomenon could not be produced by vibrios which had been killed by heat (56°C) or by alcohol.

Bernard and his colleagues maintained in this connexion that haemolysis of *V. El Tor* was facilitated by an optimum relation between the number of organisms and that of erythrocytes, the violet decoloration remaining absent in this case. Both an excess and too small an amount of blood corpuscles retarded haemolysis. The violet decoloration was marked in the former case, but disappeared as soon as lysis took place.

In a third communication (1939c) these authors stated that

1. the exohaemolysin of *V. El Tor* was inactivated by heating for 1 hour at 56°C or for 5 minutes at 100°C; was destroyed by ether or formaldehyde and neutralized by cholesterol, but not affected by toluene and activated by lecithin (egg yolk);
2. the endohaemolysin of *V. cholerae* acted slowly, producing the brown colour of methaemoglobin, was destroyed by 5 minutes heating at 100°C, neither inhibited by cholesterol nor activated by lecithin;
3. mixtures made in varying proportions of (a) the endohaemolytic substance of *V. cholerae* inactivated by heating, and (b) the active exohaemolysin of the El Tor vibrio were apt to inhibit the haemolytic action of the latter organism. Haemolysis was apt to take place, if an excess of El Tor haemolysin was used, but led to the production of methaemoglobin.
Bernard, Guillerm & Gallut reached the tentative conclusion that cholera and El Tor vibrios possessed a common haemolysin which was free in the latter organisms, combined with a neutralizing substance in *V. cholerae*. They noted that with the aid of acetone one could extract from both organisms a haemolytic substance which was soluble in ether and warm alcohol, insoluble in benzene, and thermostable when heated for 10 minutes at 100°C. Emulsified in saline at a pH of 7.2 to 8.0, it haemolysed living vibrios. This substance, which consisted of water-insoluble fatty acids, appeared to play no role in the usual phenomena of haemolysis.

In analogy with the above findings, Read, Pandit & Das (1942), through an exhaustive investigation of 62 strains of classical cholera, El Tor and cholera-like strains already referred to in Chapter 3, reached the following conclusions:

"[a] The strains can be divided into two groups: the 'early haemolytic' and the 'late haemolytic' organisms, corresponding to Greig-positive and Greig-negative organisms. In the former group, haemolysis is usually complete within a few minutes to 24 hours, is not markedly affected by performing the test at 12°C or under reduced oxygen tension and production of the haemolysin is not affected by exclusion of oxygen. In the latter group, haemolysis is usually partial, hardly occurs in 24 hours and is abolished when the test is performed at 12°C or under restricted oxygen supply.

"[b] Antihaemolytic sera prepared from Greig-positive organisms have a definite specific neutralizing effect on the haemolysin of the early haemolytic group. No similar effect has been demonstrated in the late haemolytic group."

In the opinion of Read and his colleagues, the "early" haemolysins were most likely identical with van Loghem's exohaemolysin, the "late" haemolysins with the haemodigestive ferment described by this worker.

Investigating recently three El Tor strains, one identical strain from Celebes, and 10 strains of water vibrios, all but two of which fell into Heiberg's carbohydrate group I, Brück & Brandis (1953) found that all these growths produced a soluble haemolysin, demonstrable in Berkefeld filtrates, but not in Seitz filtrates of 3-day-old broth cultures. Though thermodurable upon prolonged heating at 50°C, application of this temperature for a time sufficiently long to kill the vibrios (e.g., for 15 minutes) did not inhibit the action of this haemolysin for sheep erythrocytes. Likewise, the haemolysin was not inhibited by ultrasonic vibration sufficiently intense to kill the vibrios.

As already referred to in the preceding chapter, Zimmermann (1934) was able to establish that most of the classical cholera strains examined by him were capable of producing thermodurable haemolysins for human erythrocytes, whereas the El Tor haemolysins were active not only against these but also against sheep red blood corpuscles. As noted, these findings have been recently confirmed by De and co-authors (1954) through an examination of 27 cholera, 2 El Tor, and 14 cholera-like strains. It was found in the course of this investigation that calcium, while inhibiting the
haemolytic activity of El Tor and cholera-like vibrios, was essential for that of *V. cholerae*.

When trying to evaluate the above-discussed observations in conjunction with those described in the third chapter, one may claim that, as far as their behaviour in blood-containing media is concerned, not merely quantitative, but distinctly qualitative differences exist between the classical cholera and the El Tor vibrios. Since, however, the *V. cholerae* is to some extent endowed with haemolytic properties, it appears to be at the same time more a question of personal bias than of factual evidence whether, as far as their behaviour in blood-containing media is concerned, it is justified to place the two organisms into two different species instead of considering them as variants of one species.

**Agglutination**

**(A) Identification of suspect strains**

As can be gathered from the publication of Gruber & Durham (1896), these pioneer workers used for the identification of suspect strains with the aid of agglutination tests sera prepared by intraperitoneal immunization of guinea-pigs with killed cholera cultures. Dilutions of these sera in broth and broth suspensions of the organisms to be tested were used for both microscopic and macroscopic examinations. To carry out the former, drops of the diluted sera and of the suspensions were mixed on cover-glasses and the latter were mounted on hollow slides. Macroscopic observations (checked if necessary under magnification) were made by mixing 0.5-ml quantities of the diluted test sera and of the vibrio suspensions. Moreover, a microscopic preliminary test (*Vorprobe*) was recommended for which, instead of drops of suspensions made from pure agar cultures, loopfuls taken from the top-most layer of the primary stool cultures in fluid media were used after the presence of motile organisms had been ascertained. As stated by Gruber & Durham, it was possible under favourable conditions to obtain in this manner a fairly conclusive result within 6 to 10 hours.

Systematic studies in the Berlin Institute for Infectious Diseases led to a considerable refinement and a standardization of the agglutination technique. As summarized by Kolle & Gotschlich (1903), it was found necessary to use for the manufacture of immune sera rabbits or donkeys in preference to horses or goats, the normal serum of which agglutinated cholera vibrios at a higher titre (1:40-1:50) than the rabbit and donkey sera, the titres of which were 1:10 and 1:20 respectively. It was also indispensable to use instead of broth, apt to give inconstant results in successive tests, normal saline for the dilution of the sera and for suspension of the test organisms. Though, as stated in an instruction for the laboratory diagnosis of cholera by Koch et al., originally issued in 1902 and revised
in 1904 and 1907 (see text reprinted by Kolle & Schürmann, 1912), it was permissible to use hanging-drop preparations (inspected at low magnification and not under oil immersion) as well as macroscopic tube tests for this work, results obtained with the former method could be considered final only if quite clear-cut (über allem Zweifel eindeutig—Kolle & Gotschlich, 1903).

A modification of the agglutination technique introduced soon after these studies was the use of rapid slide tests for the preliminary identification of cholera-suspect colonies or growths. As Costa (1912) claimed, without furnishing a reference, Salimbeni was the first to take advantage of this expedient and now amply used procedure in cholera diagnostic work. To judge from a remark made by Sierakowski (1920a), an early recommendation of this method was also made by Bujwid.

Like Gruber & Durham, Dunbar (1905) suggested a rapid method for the serological identification of *V. cholerae*, according to which mucous particles of suspect stools, emulsified in drops of peptone water on cover-slips, were mixed with drops of diluted (1/500) high-titre cholera serum or, for the purpose of control, with normal rabbit serum, diluted 1/50. As claimed by Dunbar, in positive cases the motility of the vibrios was soon inhibited by the specific serum or it was even possible to observe agglutination. However, as stated by Kolle & Schürmann, tests made in the Berlin Institute for Infectious Diseases failed to confirm the value of this method. It was possible to obtain occasional positive results through agglutination tests with drops which had been removed from the peptone water cultures used for preliminary enrichment after 5 hours’ incubation, i.e., by a method analogous to the Vorprobe of Gruber & Durham. Similar procedures were also recommended by several subsequent workers without reference to the original method of these two observers.

An alternate method recommended by Bandi (1910) for the rapid laboratory diagnosis of cholera was preliminarily to add adequate amounts of specific serum to 5 ml peptone water samples filled into special drawn-out test-tubes. If these were then inoculated with cholera-suspect stools and incubated at 37°C, according to Bandi’s observations in positive cases clumps of agglutinated vibrios could be seen in the lowest part of the tubes after 2-7 hours.¹

The usefulness of Bandi’s method or of similar procedures for the rapid serological diagnosis of cholera has been endorsed by several observers. Thus as late as 1951 Cossery made the following statement:

“I have used Bandi’s test routinely for the examination of cholera-suspect vibrios since 1918.

¹ It is of historical interest that Achard & Bezaude (1897), besides making direct agglutination tests, used a method similar to that later recommended by Bandi to demonstrate the presence of agglutinins in the sera of cholera patients by cultivating cholera vibrios in 10 drops of broth to which 1 drop of serum of the patient in question had been added. As noted later, an analogous method was also applied by Ransom & Kitschima in 1898.
"During this long time I have used it in many thousands of cases. It has always given me a fairly satisfactory result if the two following conditions are observed:

"(1) A high-titre serum is used, of which the final dilution in the peptone solution is about 1:100;

"(2) The tubes are not shaken while in the incubator or while getting them out for reading."

Parallel tests made by Ghosal & Paul (1951, 1952) with the aid of Bandi's method and with two highly specific media now available—which, as will be described in a later chapter, give fully satisfactory results if used for direct platings from cholera-suspect stools—failed to confirm the outstanding value of the former test. As established under experimental conditions, Bandi's test was useful in the diagnosis of cholera when _V. cholerae_ preponderated in the stools, whereas a preponderance of coliform organisms exerted an adverse effect upon the results. In the actual examination of 285 stool samples the cultural method was found to give 38% more positive results than Bandi's method.

Considering this evidence, the WHO Expert Committee on Cholera (1952)

"came to the conclusion that Bandi's test did not give sufficiently reliable results to recommend its adoption for the laboratory diagnosis of cholera". [Page 4]

Studying 81 cholera and 31 El Tor strains, Gispen (1937, 1939) found that the O-agglutinability of alkaline saline suspensions of the former organisms which had been heated for three hours at 56°C, became as a rule inhibited or at least markedly reduced, whereas El Tor suspensions did not exhibit such a thermolability. The agglutinability of the cholera vibrios could be restored by prolonged heating or by the addition of broth or peptone to the heated suspensions. In Gispen's opinion the differences in agglutinability observed by him could be ascribed to the presence of different proteins in the cholera and El Tor vibrios respectively according to the observations made by Linton (1935). 1

De Moor (1939) expressed doubts regarding the diagnostic value of Gispen's reaction, stating that it

"may show up striking differences in a great number of cholera and El Tor strains, but the estimation of it in a given case is more difficult than Gispen pretends. Gispen mentions cholera strains that present the phenomenon either not or less distinctly . . . It appeared here that cholera strains which one time had become in- or hardly agglutinable, would another time lose hardly any of their agglutinability in the O-cholera antisera Inaba or Ogawa."

As recorded in 1948 by de Moor's assistant, Tanamal, a difference between cholera and El Tor vibrios could be demonstrated by using a potent cholera O serum, free from preservatives and diluted 1/200, to

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1 As stated in a footnote to an article by de Moor (1949), F. H. Meyer claimed in a thesis published at Amsterdam in 1939 to have observed differences in agglutinability identical with those recorded by Gispen, when treating suspensions of cholera and El Tor vibrios with 2% chloroform.
which 0.3% of sodium carbonate had been added. Cholera vibrios, if added to such a serum in a dense suspension in distilled water, did not become agglutinated, while the agglutinability of El Tor vibrios was not impaired by addition of the chemical.

It is of interest to add that, as also noted by Tanamal, cholera vibrios added in a dense suspension to a 0.5% solution of sodium bicarbonate, to which after 15 minutes an equal volume of 0.5% mercuric chloride was added, became precipitated, whereas El Tor vibrios remained in suspension.

Sharing the opinion of Gispen, de Moor (1949) ascribed the above-mentioned differences between cholera and El Tor vibrios to the different nature of their protein fractions.

(B) Tests with human sera

As noted already, Achard & Bensaude reported, soon after Gruber & Durham had recommended agglutination tests for the identification of unknown cholera strains, that this method could be used as well to demonstrate with the aid of known cholera cultures the presence of agglutinins in the sera of cholera patients.

The initial observations of these two workers have been followed up and amplified by numerous investigators, who have devoted attention to the presence of agglutinins not only in the sera of cholera patients and convalescents but also in normal persons, including those who had been vaccinated against cholera, and in carriers. The results of these studies may thus be summarized:

(1) Normal (cholera-free) individuals. As summarized by Karwatzki (1906b), and Greig (1915), in the experience of the earlier workers the normal sera of cholera-free and non-vaccinated individuals almost invariably agglutinated the *V. cholerae*, if at all, at titres not exceeding 1:10 or at most 1:20. Exceptions to this rule have remained extremely rare, Krishnan & Dutta (1950) being apparently the only observers definitely stating that they had observed once a higher titre (1:80) in a “normal” group of 18 persons tested before they received cholera vaccination. Three other members of this group showed agglutinating titres of 1:10 only, while the sera of the 14 failed to agglutinate either Inaba or Ogawa suspensions.

(2) Cholera patients and convalescents. The following of the fairly numerous observations on the presence of agglutinins in the sera of cholera patients or convalescents deserve attention for the purposes of the present disquisition.

Achard & Bensaude, reporting in 1897 on a total of 14 observations, stated that they had found agglutinins in 13 of these instances—12 times during the stage of attack (first to sixth day), when the agglutination titres ranged from 1:25 to 1:50, and once in a convalescent examined for the first time on the 28th day after the onset of cholera and then showing a titre of 1:120.
The first studies on somewhat larger groups of cholera patients or convalescents seem to have been published in 1909 by Amako, Kopp and Svenson.

Amako (1909) tested the sera of 58 cholera-affected individuals with the aid of tube tests, adding 2 mg respectively of a cholera culture to 3 ml of the various serum dilutions, and reading the results after an incubation at 37°C for 3 hours. While results during the first week after onset were negative, the agglutination titres became maximal during the second week and then decreased. The titres ranged in slight cases from 1:40 to 1:80, in moderately severe cases from 1:20 to 1:640, in severe cases from 1:160 to 1:640. No agglutination was observed in cases ending fatally or in comatous patients.

Kopp (1909), studying 32 patients admitted to a hospital in St. Petersburg, Russia, obtained positive results with agglutination tests in 26, the titres usually ranging from 1:10 to 1:50, but rarely reaching 1:100 and usually becoming maximal in the second or third week after onset. No relation seemed to exist between the character of the attacks and the height reached by the agglutination titres.

Svenson (1909), testing 37 sera of cholera patients or convalescents with a technique similar to that of Amako, but taking readings already after incubation for one hour and again after the tubes had been kept at room temperature overnight, obtained only 13 positive results. The agglutination titres, which seemed not to be influenced by the character of the cholera attacks, remained invariably low, reaching or approaching 1:50 in 8 instances, 1:25 in 5 instances. Positive results were obtained with specimens taken between the fifth and 60th day after onset of the disease, but only in rare instances before the 10th day. Evaluating his own and Kopp's results, Svenson emphasized that the agglutinatory power of the sera from cholera patients or convalescents is apt to be low and frequently not different from that of normal human sera.

In contrast to this postulation of Svenson, some other of the earlier workers observed occasionally that the sera of cholera convalescents agglutinated V. cholerae at higher titres, the maxima recorded by Liveriato (1914) and Kabelik (1915) being 1:5000.

Salimbeni (1915), examining 27 cholera patients or convalescents, laid stress upon the fact that the sera of six of these individuals, who could be tested during or immediately after the attack, had neither agglutinating nor protective properties. He concluded, therefore, that recovery from cholera took place before antibodies had appeared in the blood-stream. However, though this postulation deserves attention, it has to be kept in mind that some of the workers mentioned above as well as some quoted below noted in part an increased agglutinin response during or soon after the acute stage of the disease.

Commencing important observations on the presence of specific agglutinins in the sera of cholera patients or convalescents, Greig (1913a) found that two convalescents who continued to harbour cholera vibrios in their stools for considerable periods also produced positive agglutination reactions with their sera, whereas results of the latter test were negative in convalescents whose stools had become free from V. cholerae.

In 1915, Greig published the results of an examination of 363 sera derived mainly from cholera patients and convalescents, and to a lesser extent also from individuals in the stools of whom no V. cholerae had been found. To carry out agglutination tests with these sera, mixtures were made in capillary tubes of equal amounts of the serum dilutions to be
PROBLEMS IN IMMUNOLOGY

259

tested and of suspensions of cholera vibrios in 0.85% saline, the results being read after an incubation at 37°C for two hours. It was established through comparative tests that results of the agglutination tests were identical regardless of whether standard cholera strains or the homologous vibrios isolated from the convalescents in question were used.

The outcome of Greig's large-scale study may thus be summarized:

<table>
<thead>
<tr>
<th>Nature of sera tested</th>
<th>Number of sera</th>
<th>Result of agglutination tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>From fatal cholera cases</td>
<td>64</td>
<td>Agglutinins absent in about half of these individuals even though some of them survived for 4 to 12 days. In the 23 positive cases agglutinins appeared comparatively late and almost invariably the titres did not exceed 1:40.</td>
</tr>
<tr>
<td>From recovering cholera patients</td>
<td>210</td>
<td>Agglutinins appeared rapidly, in some instances as early as the second or third day of illness, and became as a rule well marked by the sixth day from onset. The titres, which varied from 1:400 to 1:1000, remained high up to the 17th day. As far as could be gathered from scanty observations, a drop became marked about the 20th day.</td>
</tr>
<tr>
<td>Patients from whose stools both cholera and cholera-like vibrios had been isolated</td>
<td>18</td>
<td>Agglutination was positive only with <em>V. cholerae</em> but not with the cholera-like vibrios in question.</td>
</tr>
<tr>
<td>Individuals in whose stools only cholera-like vibrios had been found</td>
<td>35</td>
<td>Agglutination tests with the homologous cholera-like strains were negative but in some instances positive results were obtained with <em>V. cholerae</em>, the individuals in question having obviously suffered from an unrecognized cholera attack.</td>
</tr>
<tr>
<td>Individuals in whose stools no vibrios had been found</td>
<td>36</td>
<td>Apparently for the reason stated above, some positive results were obtained in agglutination tests with <em>V. cholerae</em>, the titres at which reactions took place in this and the preceding group never exceeding, and but exceptionally reaching, 1:100.</td>
</tr>
</tbody>
</table>

Though realizing that agglutination tests were of little, if any, value for the diagnosis of acute cholera attacks, Greig stressed on the basis of the above-recorded observations the importance of this method for a retrospective diagnosis of the disease.

Shiiba & Oyama (1920), making agglutination tests with the sera of 97 convalescents, found that as a rule a higher titre was reached after 2-3 weeks in the Japanese convalescents (1:80 to 1:640) than in the Chinese tested, in whom the titres ranged from 1:40 to 1:160. Only 7 out of the 97 persons composing this series had titres above 1:1000 with a maximum of 1:5120 in one instance.
Tagami & Watanabe (1920), testing a series of 91 cholera convalescents, found agglutination to become positive in 87%, usually between the third and tenth day after the onset of illness, rarely later up to the 15th day. The agglutination titres ranged usually from 1:100 to 1:400, but exceptionally high titres, up to 1:10000, were said to have been met with occasionally. Individually, the highest titres were usually reached in 1-2 weeks, then a decrease set in, which was gradual at first but became more rapid after a week so that as a rule agglutination became negative one month after onset of the disease.

As referred to before, Ukil (1928) found that 25 out of the 30 cholera convalescent sera examined by him reacted positively in agglutination tests, the maximal titres being 1:100 in six instances, 1:500 in nine, and 1:1000 in ten. Supplementing this information in 1930, Ukil & Guha Thakurta stated that 15% of their convalescent sera failed to agglutinate V. cholerae, while 20% reacted at titres below 1:800, 45% at titres ranging from 1:800 to 1:1600, and 20% at a titre of 1:3200.

The studies on the agglutination reactions observable in cholera patients and convalescents by Pasricha, Chatterjee & Paul (1939) are of particular value because, in contrast to the previous investigators, these workers could base their observations on the recent findings made in regard to the antigenic structure of V. cholerae and the importance of the O antigens in immunological reactions.

Pasricha and colleagues used for their agglutination tests, made in Dryer’s tubes, suspensions of young cholera cultures to which 0.2% formol had been added as H antigens, and boiled saline suspensions of V. cholerae as O antigens. Final readings were taken after the tubes had been kept for 18 hours at 55°C in a water-bath.

Results of H and O agglutination tests made in this manner with the sera of 175 cholera patients (bacteriologically confirmed and non-fatal cases) were recorded by Pasricha and his colleagues as follows:

<table>
<thead>
<tr>
<th>Day of illness</th>
<th>Number examined</th>
<th>Number with cholera agglutinins</th>
<th>Percentage showing H agglutinins</th>
<th>Percentage showing O agglutinins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2nd</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3rd</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4th</td>
<td>8</td>
<td>4</td>
<td>50</td>
<td>12</td>
</tr>
<tr>
<td>5th</td>
<td>8</td>
<td>4</td>
<td>50</td>
<td>37</td>
</tr>
<tr>
<td>6th</td>
<td>10</td>
<td>5</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>7th</td>
<td>13</td>
<td>8</td>
<td>61</td>
<td>46</td>
</tr>
<tr>
<td>8th</td>
<td>20</td>
<td>18</td>
<td>90</td>
<td>55</td>
</tr>
<tr>
<td>9th</td>
<td>24</td>
<td>18</td>
<td>71</td>
<td>60</td>
</tr>
<tr>
<td>10th</td>
<td>23</td>
<td>17</td>
<td>74</td>
<td>57</td>
</tr>
<tr>
<td>11th</td>
<td>9</td>
<td>8</td>
<td>90</td>
<td>66</td>
</tr>
<tr>
<td>12th</td>
<td>11</td>
<td>9</td>
<td>81</td>
<td>73</td>
</tr>
<tr>
<td>Total</td>
<td>175</td>
<td>91</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>
It will be gathered from this table that

(1) No agglutinins could be demonstrated in the sera of 49 patients of this series examined during the first three days of illness.

(2) Becoming first manifest in the patients seen on the fourth day of illness, agglutinins were present in an on-the-whole increasing number of the sera from the seventh day of illness onwards.

(3) O agglutinins appeared more tardily and were throughout the observation period present in the sera in a lesser percentage than the H agglutinins.

It is in accord with this last observation that, as shown elsewhere in the article by Pasricha, Chatterjee & Paul, the maximal titre at which O-agglutination took place was 1:320 as against 1:640 in the case of H-agglutination and that the average titres of agglutination were considerably lower than those of H-agglutination.

Further interesting points elicited by these workers were that:

(a) In contrast to the postulation of Greig, agglutinins were found to be better developed for the homologous strains (isolated from the faeces of the patients in question) than for the standard Inaba strain of V. cholerae used, agglutinins for the latter being absent in 11 of the 58 sera examined in this respect.

(b) However, in 9 bacteriologically proven cases agglutinins, almost exclusively of the H type, were found for the standard strain, and no agglutinins for the homologous strains—a phenomenon for which "no satisfactory explanation" could be advanced.

(c) In conformity with Greig's findings, in patients showing both cholera and cholera-like vibrios in their stools, agglutinins were demonstrable only for V. cholerae. Such agglutinins were also found in a few patients harbouring only cholera-like vibrios in their stools as well as in some patients with clinical signs of cholera but negative bacteriological findings.

In regard to the last-mentioned point, it is, however, important to add that, according to findings recorded in the 1941 report of the Indian Research Fund Association, in some instances where both cholera and cholera-like vibrios had been isolated from the stools, agglutinins for both kinds of organisms could be demonstrated in the sera of the patients in question.

Referring to further observations on the appearance of O antibodies in the sera of 41 recovering cholera patients (23 of whom had been immunized against the infection), Rainsford (1952) stated that

"In the non-immunized group it was found that detectable O antibody seldom appeared before the fifth day and the same applied to those of the immunized group who had no detectable O antibody when they first came under observation. In both groups patients were encountered who never produced O antibody in detectable amounts throughout their illness. In the majority of cases that came under treatment early, recovery took place several days before O antibody was detected in their sera."
Rainsford concluded from these findings and from observations on the duration of the diarrhoea and the length of the vibrio excretion that "the presence or absence of O antibody had little influence if any on the presence or absence of the vibrio in the stool and was not essential or related to recovery".

The important aim of the investigations of Yacob & Chaudhri (1945) was to establish for how long agglutinins persisted in the sera of cholera convalescents—a subject which, in spite of its great importance for a retrospective diagnosis of the disease, had received but little attention in the past owing to the difficulty of observing the convalescents for prolonged periods. Yacob & Chaudhri procured for this purpose sera of persons who had suffered from cholera during epidemics taking place in the three Indian localities in question some months before.

Describing their technique, the two workers stated that they mixed 1/25, 1/50, 1/100, 1/150 and 1/200 dilutions of their sera in normal saline in Dreyer agglutination tubes with equal amounts of a suspension of live cholera vibrios, which had been found to react positively with an Inaba O serum. The tubes were kept in the water-bath at 56°C for 2 hours and then transferred to a refrigerator, readings being taken 24 hours after setting up the test. Results were confirmed by slide-agglutination tests.

The findings made by Yacob & Chaudhri may be summarized as follows:

<table>
<thead>
<tr>
<th>Locality</th>
<th>Interval between end of outbreak and date of test</th>
<th>Number of specimens examined</th>
<th>Number of specimens found positive</th>
<th>Titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kasur town</td>
<td>about 2 months</td>
<td>11</td>
<td>6*</td>
<td>1:50 to 1:150</td>
</tr>
<tr>
<td>Narli village</td>
<td>102 days</td>
<td>11</td>
<td>6</td>
<td>1:25 to 1:150</td>
</tr>
<tr>
<td>Lahore city</td>
<td>about 3 months</td>
<td>15</td>
<td>1**</td>
<td>1:100</td>
</tr>
</tbody>
</table>

* The interval elapsing between the dates of attack and of serological examination varied from 60 to 64 days.

** Tested 113 days after the time of attack.

Yacob & Chaudhri concluded, therefore, "that agglutinins can persist in the blood of recovered cases of cholera up to a period of three-and-a-half months and possibly more"—a postulation supported by the observations of some earlier workers, such as Kabelik (1915).

(3) Healthy carriers. While a number of observers, such as de Bonis (1912) and Sano (1921), found specific agglutinins to be absent or practically absent in the sera of healthy cholera carriers tested by them, positive findings have been recorded in this respect by some other workers, for instance—as has been mentioned above—by Massaglia (1911) and Levi della Vida (1913), and also in a few instances by Shiiba & Oyama (1920). As quoted by Takano, Ohtsubo & Inouye (1926), Sakai (1917) found that one-third of the 84 sera of healthy carriers tested by him aggregated V. cholerae at titres over 1:200, the highest titre observed in this series being 1:2000. As a rule, the individual carriers showed maximal titres from
four to nine days after detection of cholera vibrios in their stools, but sometimes the highest titre was reached already on the day of the first positive findings in the faeces or as late as almost two weeks afterwards. Agglutination became negative on the average within 15 days but the reaction could remain positive for 32 days. There was no definite correlation between the length of vibrio excretion by the carriers (which varied from one to 21 days with an average of eight days) and the height of the titres, but as a rule these were highest in the carriers with the longest excretion periods.

(4) Vaccinated subjects. After early observations by Bertarelli (1905), Karwatzki (1906b), and Serkowski (1906) had shown that cholera vaccination led to a marked agglutination response in the sera of the individuals concerned, this problem was further studied by numerous observers. The following of the earlier findings recorded in this respect deserve particular attention:

Balteano & Lupu (1914) concluded from rather limited experiences that:

- [a] In individuals who had received a single injection, agglutinating power becomes manifest already after 24 hours (1:20). 3 days afterwards this falls to 1:10, to raise again at the end of 48 hours. It reaches a maximum (1:130) after 24 days, then decreases gradually to 1:100 and remains at this level still two months after injection;

- [b] A similar evolution takes place in individuals who received 2 or 3 injections, except that then the titre reaches 1:150 and remains at this level for 7 days before it begins to decrease."

It has to be added that soldiers in the field who had been vaccinated, retained an agglutinating power of 1:40 for 3½ months. At the end of 5 months their titre was not higher than 1:20-1:30.

Castelli (1917) noted that, while administration of the usual cholera vaccines as well as that of cholera nucleoprotein led to an increase of the bacteriolytic titre in the sera of the vaccinated, an agglutinin response was observable only if the usual vaccines had been administered, but not after nucleoprotein had been given.

Sierakowski (1920a), studying a total of 259 individuals, to whom differently prepared cholera vaccines had been administered, established that differences in the methods of killing these products exerted a marked influence on the agglutinin levels observable in the various groups after vaccination. He further found that (a) in three persons tested before and 48 hours and 96 hours, respectively, after vaccination, higher agglutination titres became manifest within two days after the vaccine had been given; (b) in a group of 13 persons the agglutination titres, which had reached values observable in normal persons 6 months after the administration of two vaccine doses, were found to have risen 5-7 days after two booster doses had been given, without, however, reaching higher values than after the original two injections; and (c) 6 months after administration of the booster doses the agglutination titres had sunk once more to levels observable in non-vaccinated individuals.

In the experience of Sano (1921), the agglutination titres in cholera-vaccinated persons reached a maximum in 3 weeks and fell in 10 months to the levels found in non-vaccinated controls.

Hetsch (1928), summarizing the results of these and other observations made during and soon after the First World War, stated that though as a rule an agglutinin response was elicited in the sera of cholera-vaccinated
individuals, which became maximal after 2-4 weeks and remained manifest for 6-10 months, or according to some workers only for 3-4 months, the titres found varied considerably. Hetsch was inclined to ascribe these divergent results on the one hand to variations in the dosage in which the vaccines had been given and to the number of injections administered, on the other hand to differences in the technique of the agglutination tests. However, the observations of Sierakowski (1920) and further experiences referred to below leave no room for doubt that differences in the antigenic value of the various cholera vaccines exert a profound influence on the agglutinin response following their administration. It is certain at the same time that the height of the titres is markedly influenced by differences in the agglutination technique. Particularly, as stressed by Ionesco-Mihaiesti & Ciucu (1916) and confirmed by further experiences, fully satisfactory, and therefore comparable, results can be hoped for only when live cholera vibrios and not killed antigens are used for agglutination tests with the sera of the vaccinated.

Recent contributions to the knowledge on the problem presently under review were as follows:

Griffitts (1944), to whose observations on the appearance of mouse-protective bodies in the sera of cholera-vaccinated individuals reference will be made later, also noted that agglutinins appeared in these sera one week after vaccination, remained at high titre (1:180 to 1:1620) for 1-2 weeks and then diminished, the majority of the sera reacting at low titre 6 months, one year and 18 months after vaccination.

Eisele et al. (1946), testing the sera of (a) 7 individuals who had been inoculated twice with a standard cholera vaccine, and (b) 27 persons injected twice with different doses of an experimental vaccine which contained mainly the O antigen of V. cholerae, found that specific agglutinins became demonstrable at an equally high titre in both groups and also noted that administration of larger doses of the experimental vaccine did not lead to an increase of the agglutination titres.

Observations made by Gohar & Makkawi (1947) during the 1947 Egyptian outbreak showed that the sera of individuals who had been given a vaccine prepared from the autochthonous Inaba subtype cholera strain, agglutinated this as well as classical Inaba strains to a titre of 1:160, and Ogawa strains to half that titre (1:80).

In the course of interesting investigations, to which further reference will be made below, Singer, Wei & Hoa (1948b) studied the presence of agglutinins in the sera of 211 cholera-vaccinated individuals 10 days after completion of the immunization. Administration of four different types of vaccines led to a fairly high agglutinin response, the titres often reaching, and sometimes exceeding, 1:320. While the methods of producing these various vaccines did not seem to exert an appreciable effect on the titres which were attained, the agglutinin content of the sera from subjects vaccinated by the intracutaneous route was found to be significantly higher than was the case in the subcutaneously vaccinated individuals.

Ahuja & Singh (1948), to whose observations on 21 persons given 1 ml of cholera vaccine reference has been made before, found that, in marked contrast to the rise of the bactericidal titre in these individuals, the agglutination titres did not become considerably heightened, the maxima reached on the 10th day after vaccination being 1:125 to 1:250 as compared with a pre-vaccination level of 1:25.

Erdim (1951), examining the sera of 67 individuals 8 weeks after they had been given two doses of a cholera vaccine containing 8000 million of organisms per ml, found even
lower agglutination titres with a maximum of 1:160 in 46 of these individuals, 21 giving negative results.

Observations by Brounst & Maroun (1949) showed that:

(a) in a group of 371 individuals, inoculated once with 2000 million of an Inaba-Ogawa cholera vaccine and tested 30-60 days afterwards with formalized suspensions (2 per 1000) of *V. cholerae*, agglutination titres ranging from 1:25 to 1:200 were demonstrable six times only, 365 of these sera failing to produce agglutination even at a titre of 1:25;

(b) 95 of these individuals, who received 50 days after the first vaccination two booster doses totalling 12 000 million *V. cholerae* and whose sera were tested as described above, gave similarly disappointing results, agglutination at titres ranging from 1:25 to 1:100 being found only in 11 instances;

(c) somewhat better results were obtained when suspensions of live cholera vibrios, cultivated for 24 hours at 37°C, were used instead of formalized suspensions for the tests: the sera of 20 individuals, vaccinated and examined as the persons composing group (b), yielded 7 positives (i.e., 35%), the titres ranging from 1:25 to 1:200.

Further comparative tests by Gallut & Brounst (1949) with the sera of 18 individuals, vaccinated and examined as those of the above groups (b) and (c), confirmed that, except when testing unknown cholera strains with the aid of high-titre rabbit sera, it was not permissible to use formalized *V. cholerae* suspensions for agglutination tests. While obtaining as unfavourable results with the latter suspensions as Brounst & Maroun, Gallut & Brounst recorded 55% and 77% positive results when testing their 18 human sera with live suspensions of an Inaba and an Ogawa strain respectively, the titres ranging from 1:20 to 1:500.

It will be noted that these findings fully support the conclusion reached in 1916 by Ionesco-Mihaiesti & Ciucu.

(5) **Test vaccination for retrospective cholera diagnosis.** At a meeting of the Joint OIHP/WHO Study Group on Cholera held in 1949. Krishnan & Dutta (1950) recorded the following results obtained when administering 1 ml cholera vaccine to the individuals enumerated below and testing the agglutination titre of their sera two weeks afterwards:

<table>
<thead>
<tr>
<th>Number tested</th>
<th>Agglutination negative</th>
<th>1:20 or less</th>
<th>1:80 or less</th>
<th>1:520 or less</th>
<th>1:1280 or less</th>
<th>1:2560 or less</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal group</td>
<td>A. 18</td>
<td>14</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. 16</td>
<td>2</td>
<td>3</td>
<td>10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inoculated</td>
<td>A. 132</td>
<td>58</td>
<td>31</td>
<td>41</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>group</td>
<td>B. 119</td>
<td>6</td>
<td>11</td>
<td>44</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Cholera group</td>
<td>A. 17 [convalescents]</td>
<td>4</td>
<td>4</td>
<td>9</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B. 4</td>
<td></td>
<td></td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

A = before test vaccination.
B = after test vaccination.

Bearing in mind the scantiness of the evidence available in regard to the group of cholera convalescents, the Study Group recommended further observations in order to decide whether this method of test vaccination might be a useful means for the retrospective diagnosis of cholera. As was reported at the first session of the WHO Expert Committee on Cholera
in 1951, further investigations had not confirmed the usefulness of the above method for the retrospective diagnosis of individual cholera cases. It was considered possible, however, that the procedure might be helpful in establishing the nature of infection in groups of people who, giving a history of diarrhoea and vomiting, were suspected of having recently suffered from cholera. The conclusion arrived at by the Committee was

"that the method thus far had not given results of definite practical value. Should work in this direction be continued, attention ought to be given to the possible role of cross-reactions due to the presence of brucella or salmonella infections. For this reason and on general grounds great attention ought to be paid to the advisability of using in addition vibriocidal tests for the retrospective diagnosis of cholera." [Page 5]

The usefulness of the method of test vaccination for the retrospective diagnosis of cholera in groups of suspects was recently upheld by Lahiri & Dutta (1954), who drew attention to the following figures:

<table>
<thead>
<tr>
<th></th>
<th>Number tested</th>
<th>Percentage negative</th>
<th>Percentage positive at 1 : 160 or less</th>
<th>Percentage positive at 1 : 320 or above</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriologically confirmed cholera cases</td>
<td>A 94</td>
<td>2</td>
<td>46</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>B 29</td>
<td>0</td>
<td>28</td>
<td>72</td>
</tr>
<tr>
<td>Clinically diagnosed cholera cases</td>
<td>A 26</td>
<td>15</td>
<td>50</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>B 10</td>
<td>0</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

A = before test vaccination.
B = after test vaccination.

(C) Paragglutination and co-agglutination

As summarized by Sierakowski (1920b) in an important study devoted to the problem of Mitagglutination in cholera, Karwatzki (1906a) reported that he had met with some cholera-like vibrios which agglutinated at fairly high titres with cholera-immune serum, but could be distinguished from V. cholerae with the aid of Pfeiffer's reaction—results which are not surprising when the inadequacy of the sera then available for agglutination tests is considered.

Sierakowski himself found among the numerous vibrio strains isolated by him during the 1914-15 cholera outbreak in Galicia and Poland six strains of water vibrios and one derived from the faeces of a cholera convalescent, which showed morphological, cultural, and biochemical properties compatible with those of V. cholerae and agglutinated at fairly high titres with sera obtained by cholera immunization of horses, in two instances also with specific rabbit sera. However, while one of these seven strains reacted like V. cholerae in complement fixation tests, none gave a specific Pfeiffer reaction. Moreover, the sera raised against the seven strains did not agglutinate cholera vibrios and, while the latter were found to be capable of absorbing the agglutinins for the seven strains from cholera-immune sera, the reverse did not hold true, the suspect vibrios leaving the cholera agglutinins intact, if allowed to absorb cholera-immune sera.

Trying to interpret these findings, Sierakowski postulated that the agglutinating sera contained besides main agglutinins a series of minor
agglutinins (Mitagglutinine), capable of acting upon heterologous bacterial species. The latter, when brought into contact with the agglutinating sera, absorbed only the minor agglutinins, whereas the homologous organisms absorbed the main agglutinins as well. Sierakowski emphasized, therefore, the importance of agglutinin-absorption tests for a differentiation of cholera-like vibrios showing some serological relationship with *V. cholerae* from the latter organisms. It has to be stressed, however, that in the case of his as well as of Karwatzki's strains equally clear-cut differences from *V. cholerae* were revealed by Pfeiffer's reaction.

Observations indicating that organisms belonging to heterogenous bacterial genera may be agglutinated by cholera-immune sera seem to have been made first by three German workers during the First World War:

Meggendorfer (1918) isolated from the faeces of a healthy soldier a large motile bacillus which was agglutinated to full titre by cholera-immune serum, but whose homologous serum exerted no action on *V. cholerae*. Results of agglutination tests with cholera-immune serum remained unchanged after the bacillus in question had been subcultivated 74 times. In view of these findings, Meggendorfer stressed the necessity of verifying the vibrio nature of the strains to be serologically tested in cholera-diagnostic work.

As quoted by Meggendorfer, Quadflieg (1916) cultivated from the stools of a cholera suspect an *E. coli* strain which, though not reacted upon immediately, was after 4 hours agglutinated by cholera-immune serum at titres nearly corresponding to those found in the case of *V. cholerae*.

Examining roughly 1000 stool-specimens, Messenschmidt (1916) found in about 20—partly in association with cholera vibrios—bacteria belonging to the *E. coli, Proteus*, or *Sarcina* groups, which became agglutinated at full titre by cholera-immune sera and retained this property after repeated subcultivation, as far as observed for 4 months. Like Meggendorfer, he stressed the importance of a preliminary smear examination of the growths to be tested serologically, as prescribed in the official German instructions for cholera diagnosis.

Following up an earlier observation by Wong (1936) on two individuals who, to treat eye diseases, had been injected intravenously with cholera-typhoid vaccine, Wong & Chow (1937)

(a) demonstrated the presence of group agglutinins for *V. cholerae* and *Brucella abortus* in rabbits immunized by the subcutaneous or the intravenous route with either species or with cholera-typhoid vaccine;

(b) injecting six human subjects either intravenously or subcutaneously with this vaccine, found that while the sera of all six individuals agglutinated cholera vibrios at titres of maximally 1:160, 4 of the sera also agglutinated *Br. abortus* at titres of maximally 1:40.

The validity of these initial findings was fully confirmed through observations on larger groups of cholera-vaccinated individuals by Eisele and colleagues (1946, 1947, 1948) and by Erdim (1951). That the brucella agglutinins developing in the sera of such persons—often at higher titres than observed by Wong & Chow—are apt to persist for considerable periods is well illustrated by the records of Eisele et al. (1947), who demonstrated the presence of these agglutinins in 27% of the members of a group tested 18 to 28 months after cholera vaccination.
Following up and amplifying laboratory studies by Eisele et al. (1946), McCullough, Eisele & Beal (1948) reported that:

"Reciprocal agglutinin-absorption tests on antiserums for *V. comma* and the brucella species (*abortus, suis, melitensis*) showed conclusively that the antigen shared by these groups of organisms is an H antigen of *Vibrio comma*. This antigen is present in all three species of brucella with minor qualitative and quantitative differences."

In strict contrast to this conclusion, Gallut (1950) stated that the cholera vibrio and the brucella species possessed common O antigens, found to correspond to the antigenic factors C and D of *V. cholerae* for the strain of *Br. suis* tested, and to the factor D only in case of the two *Br. melitensis* strains examined. Recording the observations he made when testing these brucella strains with cholera-immune sera, Gallut noted that

"It is known that for cholera diagnosis agglutination tests must be made with unheated vibrio suspensions, preferably those of living vibrios [Gallut & Broust, 1949]. By this technique the El Tor vibrios cannot be differentiated from the authentic cholera vibrios. However, their different chemical composition (protein I of Linton for the *V. cholerae*, protein II for *V. El Tor*), produces differences in their serological behaviour after heating for 3 hours at 56°C. We submitted *Br. abortus* suspensions ... to such heating and found that these organisms behave like true cholera vibrios and not like El Tor vibrios. This has been confirmed by the use of a serum raised against the protein of *V. El Tor* which failed to agglutinate *Br. abortus suis*."

In a further paper, Gallut (1953c) stated that he had studied the question whether a pure A-type cholera-immune serum could be obtained by the absorption of a serum raised against the Inaba variant of *V. cholerae* (which according to him had the antigenic formula A C) with *Br. suis*. It was found that the latter organisms were incapable of fully absorbing the C antibody of Inaba serum.

As Gallut (1954b) added in a subsequent paper devoted to a critical evaluation of the retrospective serodiagnosis of cholera, he had been able to make the following observations on an individual who, while never having been vaccinated against that disease, had shown signs of a brucella infection, contracted in the laboratory, in October 1953:

<table>
<thead>
<tr>
<th>Species used for agglutination tests</th>
<th>Maximum titre 20 October 1953</th>
<th>Maximum titre 6 January 1954</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Br. abortus suis</em></td>
<td>1:1666</td>
<td>1:80</td>
</tr>
<tr>
<td><em>Br. melitensis</em></td>
<td>1:2000</td>
<td>1:80</td>
</tr>
<tr>
<td><em>V. cholerae Inaba</em></td>
<td>1:1000</td>
<td>1:80</td>
</tr>
<tr>
<td><em>V. cholerae Ogawa</em></td>
<td>1:100 (partial)</td>
<td>0</td>
</tr>
</tbody>
</table>

Commenting on these findings, Gallut stated:

"Thus even after three months the cholera agglutination titre was significant. It is evident that such a serodiagnosis, if made with a vibrio of the Inaba variety, indicates a retrospective diagnosis of cholera. Therefore, since the common factor is absent from the endemic Ogawa variety (AB), it seems judicious and essential to us to utilize solely suspensions of the latter variety for the serodiagnosis of cholera in cases where this is associated with a positive serodiagnosis of brucellosis."

[Trans.]
In their valuable study on the laboratory aspects of the 1947 cholera outbreak in Egypt, Gohar & Makkawi (1948) noted that on several occasions suspicious colonies which gave positive results in slide agglutination tests with a cholera O serum, were ultimately identified as _B. faecalis alcaligenes_. Following up these observations, Gohar & Makkawi confirmed not only that _B. faecalis alcaligenes_ strains were agglutinated at a titre of 1:25 by cholera O serum but established also that _V. cholerae_ was reacted upon at the same titre by immune sera raised against _faecalis alcaligenes_ strains. However, when absorption tests were made, neither these organisms nor cholera vibrios were found capable of completely absorbing the agglutinins from the heterologous sera. It appeared, therefore, that the organisms concerned "merely shared an O-antigenic fraction". Gohar & Makkawi also referred to previous observations by one of them which had shown that _V. cholerae_ shared an H-antigenic fraction with _Salmonella enteritidis_.

A further study of the antigenic relationship existing between the Inaba strains of _V. cholerae_ isolated in Egypt and _S. enteritidis_ was made by Felsenfeld (1948). He concluded from agglutination and agglutinin absorption tests made with 3 Egyptian cholera strains and various _Salmonella_ strains, respectively with Inaba H-O and _Salmonella_ O and H sera, that the _V. cholerae_ strains contained fractions of the _Salmonella_ antigens I, XII and g.

A more general investigation of the serological relations existing between _V. cholerae_ and common Enterobacteriaceae was undertaken by Felsenfeld et al. (1951). Making slide agglutination tests with cholera H-O sera, they obtained the following results with various faecal strains isolated at Chicago:

<table>
<thead>
<tr>
<th>Organism</th>
<th>Number tested</th>
<th>Number positive in agglutination tests with H-O sera raised against <em>V. cholerae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inaba</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>70</td>
<td>11</td>
</tr>
<tr>
<td><em>A. aerogenes</em></td>
<td>134</td>
<td>26</td>
</tr>
<tr>
<td><em>Paracolobactrum</em></td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td><em>Proteus</em></td>
<td>29</td>
<td>2</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>55</td>
<td>2</td>
</tr>
<tr>
<td><em>Faecalis alcaligenes</em></td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>315</td>
<td>49</td>
</tr>
</tbody>
</table>

Absorbing Inaba H-O serum with _Brucella melitensis_, _Br. suis_, _S. enteritidis_, _E. coli_, _A. aerogenes_, and _P. morgani_ strains, Felsenfeld and co-workers found

"a fraction of the cholera 'H' antigen to be identical with the factor common to the 'g.m.' antigen of _Salmonellae_ and a part of the _Brucella_ flagellar antigen, while a common 'O' antigen component was found to be part of the I, IX, XII antigen of _Salmonellae_".
Further testing the agglutinability of various *Paracolobactrum* species with cholera-immune sera, Malizia (1954) recorded the following noteworthy results:

| Antigens from | Strains tested | II-O cholera sera absorbed
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. coliforme</em></td>
<td>120</td>
<td>4</td>
</tr>
<tr>
<td><em>P. intermedium</em></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td><em>P. Bethesda</em></td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

As Malizia stated, these findings indicated

"a fairly close antigenic relationship between the *P. Bethesda* strain and the *V. comma* (Inaba) strain. Whether this similarity indicates that this strain plays a more important role in enteric infections than previously recognized warrants further study."

**Changes in agglutinability**

In order to do full justice to the problem of whether agglutinability by the usually available specific sera is an unalterable characteristic of *V. cholerae*, permanently distinguishing this organism from the cholera-like vibrios, it is necessary to scrutinize not only whether authentic cholera vibrios may lose their agglutinability by the above-mentioned sera, but also whether agglutinability by such sera may be acquired by vibrios initially giving negative reactions in this respect.

**Loss of agglutinability.** Apart from Bordet, who claimed in 1896 that animal passage could render the cholera vibrio inagglutinable with specific serum, Ransom & Kitashima (1898) seem to have been the first workers to have devoted attention to the subject presently under review. They noted that, in contrast to its agar-grown parent strain, a substrain of *V. cholerae*, which had been passed twenty times through broth containing 1% cholera-immune serum, exhibited feeble or even no agglutinating power if incubated for 24 hours in 10-ml amounts of the same medium with a specific serum content of 1%-2%.

The original observation of Ransom & Kitashima that growth in the presence of its homologous serum is apt to impair or to inhibit the agglutinability of *V. cholerae*, has been repeatedly confirmed by subsequent observers. There can be no doubt that a transition into the rough state is responsible for this well-authenticated alteration.

Some workers in Russia, particularly Zlatogoroff (1909, 1911) and Horowitz (1911), seem to have been the first to assert that *V. cholerae* was apt to lose its specific agglutinability in the human intestine or in water supplies, and a few other observers such as Barrenseheen (1909) and Puntoni (1913b) confirmed the findings made in the latter respect by Zlatogoroff. However, several other investigators, such as Haendel & Woithe (1910), Köhlish (1910), Wankel (1912), and Bindi (1913), though working in part
with the strains and even with the diagnostic serum of the Russian observers, were unable to confirm the findings of the latter. Stamm (1914) found that several of the cholera strains tested by him no longer became agglutinated with cholera-immune serum after they had been repeatedly passed through water, while others remained unaltered even after more frequent passages. The strains which were no longer agglutinated, were almost without exception still immunogenic, i.e., capable of producing immune sera positively reacting with cholera vibrios. Since in Stamm's opinion variations of V. cholerae, including changes in agglutinability, were not easily effected under natural conditions but, once they had taken place, were apt to be permanent, he declared that

"consequently it is impossible to explain the rise, cessation and recrudescence of cholera epidemics through the hypothesis of a transmutation of the cholera vibrios into saprophytic variants and vice versa". [Trans.]

Similar views were vigorously expressed by the orthodox German school, Kolle (1909b) even maintaining that any "non-agglutinable" vibrio found in human stools was not a cholera vibrio. It has to be noted, however, that this extreme view was reached at a time when the phenomena of dissociation were still unknown. Indeed, it would seem that Finkelstein (1931) was the first worker inclined to ascribe the loss of agglutinability by V. cholerae after a sojourn in water to a transition from the smooth into the rough state.

Finding that, if cholera stools were added to Indian tanks (water reservoirs), V. cholerae invariably lost its agglutinability with specific serum within 16-20 hours, Tomb & Maitra (1926) were led to consider the "agglutinating" faecal vibrios and the "non-agglutinating" organisms met with in the tanks, and also in human carriers, as identical in character. The far-reaching conclusions these two workers drew in strict contrast to Stamm's views from these and related observations will be discussed later on.

Brahmachari (1927, 1928, 1929), to whose work also further reference will be made below, maintained like Tomb & Maitra that passage through water rendered the cholera vibrios inagglutinable with specific serum and also claimed that intravenous injection of rabbits with V. cholerae led to the appearance of inagglutinable vibrios in the stools of the animals—a result which, in view of the ubiquity of cholera-like vibrios in India ought to be considered as a post hoc rather than a propter hoc phenomenon.

Minervin (1931), in order to study the changes V. cholerae was apt to undergo when introduced into specifically immunized animals, injected typical cholera vibrios into the testicles of cholera-immune rabbits and excised these organs 3-10 days later. In three instances it was possible to isolate from the excised testicles cultures of vibrios which had obviously undergone roughening. Agglutination tests with these passage strains gave at first negative results, but it was possible to restore agglutinability in one
instance through repeated subcultivation, and in the other two through animal passage (intravenous injection of rabbits).

D'Hérelle, Malone & Lahiri (1930) observed some instances of inagglutinability with specific-immune serum in the case of cholera vibrios isolated from the faeces of convalescents, and suggested that this change was the result of bacteriophage action. Referring to these observations, Vardon (1940) expressed the opinion that the corresponding changes noted by Tomb & Maitra (1926) in the case of faecal vibrios exhibited in water tanks might have been due to the same cause. Morison (1932), while admitting that "the bacteriophage has something to do with the production of rough from smooth vibrios and vice versa", added that he had "been unable in the case of cholera to make agglutinating vibrios inagglutinable by growing them in the presence of bacteriophage". However, Doorenbos (1932) adduced evidence to show that the presence of bacteriophage was apt to alter the agglutinability of cholera vibrios and it would seem that Morison (1935) obtained the same result when using instead of pure-line bacteriophage-strains combinations of different races.

Yang (1935), keeping cholera vibrios derived from one strain in the dark at room temperature in raw, candle-filtered, and autoclaved water samples respectively, found that the agglutinability of the organisms became lost in the raw and filtered river water samples as well as in the raw canal and well water samples after periods ranging from 21 to 28 days.

The present writer, on the contrary, when studying the survival of cholera vibrios in numerous filtered and autoclaved samples of Shanghai surface-waters kept under conditions identical to those used by Yang, was never able to observe a loss of agglutinability of the test organisms, even though some of the specimens could be watched for a period of almost a year. Vibrios which were not agglutinable with cholera-diagnostic sera could be isolated not rarely side by side with *V. cholerae* from the stools of patients. Since, however, cholera-like vibrios abounded in the surface waters and were occasionally met with in the stools of healthy individuals at times when cholera was absent from Shanghai, it was not possible to consider the occurrence of "inagglutinable" organisms in the cholera stools as significant. This was in accord with previous observations by Crendiropoulo (1912), who adduced evidence to show that the apparent replacement of *V. cholerae* by inagglutinable organisms in the stools of carriers was really the result of an initial co-existence of cholera and cholera-like vibrios in the intestines of these individuals.

(2) Acquisition of agglutinability. Zlatogoroff, apparently the first worker who paid systematic attention to the subject presently under review, to which some general reference had already been made by Sanarelli (1893), claimed in 1909 that he had succeeded in rendering 10 out of 18 water
vibrio strains, originally found to give negative results in agglutination tests, agglutinable with cholera-immune serum. The methods he used for this purpose were (a) frequently repeated subcultivation, resulting in the appearance of agglutinable vibrios in the 54th generation; (b) subcultivation alternating with intraperitoneal passage of the strains through guinea-pigs; and (c) combination of the latter procedure with the simultaneous injection of killed typhoid or *E. coli* cultures or of living streptococci so as to increase the virulence of the growths. Evaluating his results, which he stated that he had confirmed with the aid of Pfeiffer’s reaction in one instance and through complement-fixation tests eight times, Zlatogoroff pointed to the importance of the water vibrios as a potential source of cholera infection.

Reporting on further investigations, Zlatogoroff (1911) stated that he had been successful in restoring the agglutinability of *V. cholerae* lost in the human intestine not only with the aid of the above-mentioned methods, but sometimes also by subjecting the serologically altered vibrios, after they had been suspended in cholera-immune serum diluted with normal horse serum, to repeated freezing and thawing. The method of simply passing the vibrios through diluted cholera-immune serum seemed unreliable because of the possibility of its leading to spontaneous agglutination.

Zlatogoroff concluded from his observations that “each vibrio which is isolated from the faeces during an epidemic or at its onset, even if it does not agglutinate, should cause suspicion of cholera for the reason that the agglutinability of the cholera vibrios is very changeable.” [Trans.]

Horowitz (1911) who found besides intense subcultivation symbiosis with *Sarcina lutea* effective in restoring the agglutinability of *V. cholerae* lost in the human intestine, reached an identical conclusion.1

Several other workers, such as Köhlisch (1910), McLaughlin & Whitmore (1910), Freifeld (1912), and Wankel (1912) were not able to confirm the validity of the above-recorded findings, the last-mentioned observer stating that “even though the techniques recommended by Horowitz and Zlatogoroff have been followed most painstakingly, it was not possible to transmute even a single of the ten strains from Petersburg into an authentic cholera strain.” [Trans.]

A further noteworthy observation made by Douglas (1921) concerned a “paracholera” vibrio strain which after repeated subculture on artificial media had acquired agglutinability with cholera-immune serum. However, since suspensions of this organism were found incapable of absorbing the agglutinins for *V. cholerae* from the immune serum, the positive reactions it gave in agglutination tests were apparently not of a specific nature. It served as a corollary for this assumption that a serum raised against the paracholera vibrio failed to agglutinate *V. cholerae* even at a titre of 1:100.

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1 It should be noted that Puntoni (1913a) also claimed good success when growing a strain of “inagglutinable” vibrios five times in succession in the presence of two organisms isolated from the air.
These findings well illustrate that claims regarding the appearance of agglutinability in vibrios have to be interpreted with great caution, unless they have been supported by further and thorough serological examinations of the supposedly transmuted strains.

Tomb & Maitra (1927, 1928), though admitting that the few attempts they had made in the laboratory to render initially inagglutinable vibrios agglutinable with cholera-immune sera had given “inconclusive and inconstant” results, laid great stress upon the fact that they had invariably been able to effect a transmutation of cholera vibrios into an inagglutinable form in their water-tank experiments and also upon observations regarding the frequency of organisms of the latter type in human faeces as well as in the tanks. They felt entitled to conclude from this evidence

“that the non-agglutinating vibrio ... takes on the agglutinating characteristic under certain biochemico-physical conditions in the human intestine the nature of which are at present unknown, and in this mutation or epidemic form is the cause of epidemic cholera, since it is not unreasonable to assume that a characteristic so unstable may be as easily acquired as lost”.

Claims as faroing in their implications as those of Tomb & Maitra were made by Brahmachari (1927, 1928, 1929) and by Pasricha and colleagues (1931, 1933).

The former worker reported in 1927 that not less than 40 out of 68 strains of vibrios which had been isolated in an endemic area in Calcutta from patients showing clinical signs of cholera, healthy persons, or water tanks, and which initially did not become agglutinated with cholera-immune serum, gave positive results in agglutination tests after they had been kept for six months, particularly if they had been frequently subcultivated.

Brahmachari (1929) also claimed that he had been able to restore through animal passage the agglutinability of cholera vibrios which had become spontaneously lost in the intestine of an intravenously infected guinea-pig.

Testing the action of cholera bacteriophages on 355 cholera-like strains, Pasricha, De Monte & Gupta (1931) found that “the secondary phage-resistant colonies that develop after the action of cholera bacteriophages... in some experiments are agglutinable by cholera high-titre serum. They completely absorb the agglutinins from a cholera-type serum and produce a serum which agglutinates cholera vibrios in very high dilutions”.

Pasricha and colleagues admitted that this acquired agglutinability was difficult to maintain on subculture, requiring repeated plating and selection of the best agglutinating colonies. They felt certain, nevertheless, that a large proportion of the vibrios found in cholera-affected places, which differed from V. cholerae only in their serological reactions, were mutant forms of the latter organism and played “a great part in the aetiology of the disease”.

In their second paper (1933), Pasricha, De Monte & Gupta maintained that an action identical with that of the cholera phages was exerted by vibriophages, which were capable of lysing only cholera-like but not true cholera vibrios. They claimed in this connexion to have obtained the following results from experimenting with 56 strains of recently cholera-like vibrios:

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<td>Remained serologically unchanged after bacteriophage action</td>
<td>32</td>
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<tr>
<td>Became agglutinable after action of cholera phage</td>
<td>11</td>
</tr>
<tr>
<td>Became agglutinable after action of vibriophage</td>
<td>13</td>
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<td><strong>Total</strong></td>
<td><strong>56</strong></td>
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Yang (1935), subjecting four water vibrio strains which were originally inagglutinable with H + O cholera-immune serum, to daily subculture in plain broth and sheep-serum broth respectively, found that all samples except one subcultivated in sheep-serum broth became rather suddenly agglutinable with the specific serum after intervals ranging from two to six days.

Summarizing the results of a study on vibrios isolated from non-cholera sources in India, Taylor & Ahuja (1935a) stated that:

“A vibrio isolated from water in an area widely removed from places where cholera is endemic and which had been free from cholera for a number of years, was inagglutinable when first received, but in a period of six months' sub-culture in the laboratory developed all the biological characters of an authentic cholera vibrio, including H and O agglutination to full titre, and was indistinguishable from a cholera strain when quantitative and qualitative tests were applied.”

While stressing that this strain differed in chemical structure from typical strains of *V. cholerae*, Taylor & Ahuja stated that the epidemiological significance of vibrios agglutinable with cholera-immune serum, yet chemically differing from typical Group I cholera-vibrios, was not clear, it having not yet been determined whether the vibrios of an aberrant chemical structure might be cholerigenic.

In a second paper (1935b), Taylor & Ahuja stated that they had been able to produce through a series of intraperitoneal passages in mice agglutinable variants giving the H and O reactions of typical *V. cholerae* strains in the case of three formerly inagglutinable vibrios, namely, (1) a strain of *V. metchnikovi*; (2) a water vibrio isolated three years previously in Calcutta; and (3) a vibrio obtained in an endemic area of Bengal from a healthy person. In the case of the two last-mentioned strains the acquisition of agglutinability was accompanied by a shift in chemical constitution.

Aptly summarizing observations in point made by Linton, Shrivastava & Mitra (1935; see also Linton, 1935) White (1937b) stated that:

“From a first plating of cholera stool two colonies were picked off yielding, respectively, a typical culture of *V. cholerae* termed ‘Rangoon smooth,’ and a vibrio race, termed ‘Rangoon rough 1,’ held to be a rough derivative of *V. cholerae* and showing no serological nor antigenic relationship with that organism. From ‘Rangoon rough 1’ there was isolated a race, ‘Rangoon rough 2,’ growing in convoluted colonies and serologically distinct from ‘Rangoon smooth’ and ‘Rangoon rough 1.’ Next there was separated from ‘Rangoon rough 2’ a fourth race, ‘Rangoon rough 2a,’ described as smooth-rough, serologically intermediate between ‘Rangoon smooth’ and ‘Rangoon rough 2’; and finally from this a fifth race, ‘Rangoon smooth recovered,’ in which the distinctive serology of *V. cholerae* was completely restored.”

These shifts in agglutinability were stated to have been accompanied by shifts in the chemical composition of the successively isolated strains: while “Rangoon smooth” and “Rangoon rough 1” were found to belong to the chemical Group I of Linton and colleagues, “Rangoon rough 2”
showed the chemical characteristics of Group V, and "Rangoon smooth recovered" those of Group VI.

Commenting upon these modifications, Linton (1935) declared that either they might have resulted from successive changes in the molecular arrangement of the vibrio proteins and carbohydrates, or all the different variants might have been present in the originally isolated strain. However, in a further paper Linton, Seal & Mitra (1938) stated that they had obtained from a strain of "Rangoon rough 2," cultivated from a single cell, through 10 daily transfers in 0.5% glucose broth a variant which was inagglutinable with "Rangoon rough 2" immune serum, but was agglutinable with the serum raised against the original smooth Rangoon strain and was otherwise as well indistinguishable from the latter, showing the chemical constitution of Group I. The serological results obtained with H+O sera were confirmed through agglutination tests with an Inaba O serum which, while not producing a reaction with the "Rangoon rough 2" strain, agglutinated the smooth variant obtained from this at titres up to 1:2500.

Discussing these findings, Linton and co-authors stated that:

"Although almost nothing is known about the internal arrangement of the vibrios, it may perhaps be permissible to suggest that each of them possesses the enzymic equipment capable of synthesizing the various proteins and polysaccharides which are found in the whole group."

Yu (1940) recorded that he had been able to render 16 out of 20 water vibrio strains, which had been isolated at the time of a cholera outbreak in Shanghai from the Whangpoo river, partially agglutinable with cholera O serum by passing the organisms suspended in mucin five times in succession by the intraperitoneal route directly from guinea-pig to guinea-pig. He felt entitled to conclude from this observation that such transformations might also take place under the influence of mucin in the human intestine, particularly in the case of gastro-intestinal disturbances, when mucoid substances were apt to be abundant. It has to be emphasized, however, that Yu's strains had initially shown a trace of agglutinability with cholera O serum. Hence, as maintained by Gallut (1951), these organisms possibly contained minor O-antigenic factors apt to react with non-specific components of the O serum used. Be this as it may, Gallut (1951) obtained entirely negative results when repeating Yu's experiments with seven Egyptian water-vibrio strains, which were apparently similar in their initial serological properties to the strains of the latter worker. While Gallut's strains did not acquire any specific agglutinability when being passed in mucin suspension ten times from mouse to mouse, they lost after the third to the sixth passage the agglutinability with their homologous serum, so that mucin seemed to degrade rather than to enhance the serological properties of the organisms. Some modifications of the chemical properties became noticeable in the course of the passages but these occurred also in the vibrios passed
in mucin-free suspensions through control animals and even in the cultures of the strains kept in stock.

As will be perceived from above-recorded statements, numerous workers have claimed success in restoring the lost specific agglutinability of cholera vibrios or even in transmuting cholera-like vibrios, which originally failed to react positively in agglutination tests with cholera-immune sera, into organisms behaving in this respect like V. cholerae. However, in view of the technique adopted by them, the results recorded by most of these workers have to be viewed with great scepticism. They often failed to confirm their findings through adequate agglutinin-absorption and cross-agglutination tests. Further, it is frequently impossible to rule out the possibility that the strains with which the experiments were started were not of a uniform composition but contained, besides a large number of organisms reacting negatively in specific agglutination tests an initially unrecognized minority of true cholera vibrios. Most important finally, it must be kept in mind that with a very few exceptions the observations referred to above have been made with not fully specific H-O sera.

It is under these circumstances not surprising to find that, whenever some workers reported that they had brought about a serological transmutation of vibrios, others, even though using identical methods, failed to substantiate these claims. It has been stated already in this connexion that several investigators, repeating the experiments of Zlatogoroff and of Horowitz, were unable to confirm the findings of these two observers. It has likewise been noted that, history repeating itself, Gallut (1951) recently obtained strictly negative results when checking the validity of claims similar to those of Zlatogoroff and Horowitz, made by Yu in 1940. The papers read by Tomb & Maitra and by Brahmachari in 1927 were also much criticized, Pandit, for instance, stating that, though he had kept cholera-like vibrios isolated from water supplies in India for over two years, he had failed to note the change in the agglutinability of such strains claimed to be frequent by Brahmachari.

A most determined stand against what he called the "legend" of serological transmutability of vibrios was taken by White (1937b). Discussing in particular the above-mentioned observations of Linton and colleagues, White maintained that since

"'Rangoon smooth' and 'Rangoon rough' were derived from two colonies in a first plating of stool, belief in their genetic connection is a matter of pure assumption".

White's main objection not only to the validity of the claims made by Linton and co-authors but also to that of the results recorded by Taylor & Ahuja (1935b) was based upon tests he made with a special type of cholera bacteriophage, the LL phage (White, 1937a). He stressed that, both in the case of the Rangoon series and in that of Taylor & Ahuja's strains, cultures found to be infected with this phage were alleged to have been derived from
growths in which it was absent. Discussing this discrepancy, White said that:

"Various hypotheses may be improvised to fit the facts: genesis of bacteriophage \textit{de novo}; mutation of vibrio phage or vibrio phages unknown, collaterally with that of the vibrio itself; but the simple and obvious indication is that the alleged mutant cultures are not derived from the parents presented."

Generally speaking, White maintained that:

"There is, I believe, not only insufficient evidence on which to base a theory of vibrionic transmutability such as is at present current, but definite evidence against acceptance of such alleged instances of change as have been discussed."

Referring again to the Rangoon strains, White (1940a) stated that:

"The negative results obtained with extracts of a rugose derivative of 'Rangoon rough 1,' which is a smooth culture with the serology of \textit{V. metchnikovi}, and of the capsulated culture 'Rangoon rough 2' support my contention . . . that these strains have no immediate relation to nor derivation from their alleged parent, the cholera strain 'Rangoon smooth.'"

In view of White's objections it is difficult to assert the validity even of Taylor & Ahuja's observations. However, even if one could admit the possibility that under highly artificial conditions the agglutinatory properties of vibrios might become changed, there is no convincing evidence to show that such transmutations take place under natural conditions and that consequently cholera-like vibrios or cholera vibrios which had lost their agglutinability with the usual specific sera form a reservoir from which epidemics may be produced \textit{de novo}. It is significant to note that Seal (1935), one of Linton's principal co-workers, discussing in particular the variations brought about by bacteriophage in the laboratory, considered it an open question whether such changes in the character of vibrios "do also occur in nature or inside the human system".

A peremptory statement made in this respect by d'Hérelle (1928), when discussing Tomb & Maitra's claims, was that he could "not agree with the possibility of the regression from non-agglutinating to agglutinating."

In our quarantine station of Tor, during the last fifty years, hundreds of thousands of pilgrims harbouring non-agglutinating vibrios in their intestine have passed through the station on their way towards the North, and not a single case of cholera has been discovered amongst them nor has an outbreak of cholera ever occurred north of Tor. We must conclude that, in Nature, the regression from non-agglutinating to agglutinating vibrios does not take place and that carriers of such non-agglutinating vibrios are harmless and never the origin of an outbreak of cholera. To say that non-agglutinating vibrios may be the cause of the epidemicity is a mere hypothesis, but to show that a Mecca pilgrim carrier of non-agglutinating vibrios has never been the cause of an epidemic, that is a fact."

Gallut (1951), commenting on the significance of Yu's claims, similarly stressed that observations on the incidence of cholera in Shanghai did not support the idea of a causative role played by the water vibrios in the origin of the epidemics.
The validity of this statement is fully supported by the observations the present writer had opportunities to make in most cholera-affected parts of China during about a score of years. Though water vibrios were found to abound everywhere, not a single cholera outbreak was seen which was not found to have been due either to an importation of the infection or to its continued sporadic occurrence in man. In this sense, therefore, one is certainly entitled to consider the alleged serological transmutability of vibrios to be a myth.

**Haemo-agglutination**

As summarized by Doorenbos (1932), he observed in 1931 that, when a few drops of a suspension of sheep erythrocytes were added to a saline suspension of recently isolated El Tor vibrios, after a few minutes the colour of the blood changed to violet and at the same time the blood corpuscles began to become agglutinated in the form of small flocculi which rapidly sank to the bottom of the tubes. Further studying this phenomenon, Doorenbos established that

(a) the reaction took place at 37°C as well as at 0°C;
(b) the haemo-agglutinins were inactivated by heating the suspensions for 5 minutes at 64°C;
(c) the haemo-agglutinins were absorbed by red blood-corpuscles;
(d) some strains possessed the property of haemo-agglutination only during a short period of their development, the phenomenon suddenly appearing after a few hours’ incubation and disappearing as quickly when the cultures became older;
(e) the haemo-agglutinins inhibited the action of the haemolysins and vice versa.

Doorenbos added that seven Syrian strains, recently isolated from carriers who had arrived from a cholera-affected area (Iraq), produced a marked haemo-agglutination only in guinea-pig blood suspensions, and a feeble reaction if goat blood was used. Haemolysis tests with these strains gave more clear-cut results, proving positive with guinea-pig blood, and negative with goat blood.

Panayotatou (1931), using guinea-pig blood for such tests, had satisfactory results with four strains from Basra, haemo-agglutination becoming manifest before haemolysis became apparent. She recommended using for the former tests 4- to 6-hour-old cultures, suspending the vibrios in broth diluted 1/10, 1/100, and 1/1000 respectively with normal saline and reading the results after an incubation at 37°C for 15 minutes. She confirmed that haemo-agglutination disappeared as soon as haemolysis set in.

While in the opinion of Panayotatou haemo-agglutination tests appeared to be of value for the laboratory diagnosis of cholera, Cantacuzène (1933), finding that identical reactions were given by various cholera-like vibrios, considered such tests to be without practical importance.
Gallut & Brumpt (1944) explored the possibility of whether in cholera-diagnostic work advantage might be taken of the method of "haemo-agglutination" of Brumpt (1941), for which, instead of the serum, the whole blood of the patients was used for rapid tests on slides or gelatinized paper. Besides working with formalized H+O suspensions, Gallut & Brumpt also made tests with O suspensions of _V. cholerae_, prepared by suspending alcohol-killed vibrios after centrifugation in 10% sodium citrate solution and adding one drop of a 1% methylene blue solution per ml. The rabbits, whose blood was tested, had been immunized either with H+O or with O antigens. It was found that O as well as H+O haemo-agglutination took place within a few minutes or even seconds at ordinary temperature, while tests with the blood of normal animals or of healthy human beings gave negative results. Gallut & Brumpt therefore recommended this method, which had proved satisfactory in the case of other infections, such as typhoid, paratyphoid, and typhus, for the diagnosis of cholera. So far, however, no practical advantage seems to have been taken of this recommendation or of the proposal of Felsenfeld and co-workers (1955) to utilize for the purpose of cholera diagnosis the haemagglutination method suggested by Neter and colleagues (1952) for the recognition of enteric infections producing low serum-agglutination titres.

**Acid agglutination**

It being proposed, for the convenience of the record, to deal with acid agglutination tests at the present juncture, it has first to be noted that Beniasch (1912) and Sgalitzer (1914) found this method unsuitable for a differentiation of cholera and cholera-like vibrios. However, Vercellana (1926) recorded that, when tested with lactic acid, cholera-like vibrios became invariably and rapidly agglutinated in the form of large and stable flocculi, whereas cholera vibrios, if reacting at all, became agglutinated at lower dilutions only in the form of small and unstable flocculi. Damboviceanu (1933), testing 63 strains of _V. cholerae_ and 34 cholera-like strains with the aid of acid agglutination tests, found that more than half (58%) of the latter reacted like the cholera vibrios. However, she expressed the opinion that tests of this kind might be of value for a distinction of cholera-like strains which were descendants of _V. cholerae_ from those having no genetic relation with this organism.

**Precipitin reactions**

Though, as noted above, Kraus (1897) drew attention early to the precipitin reactions taking place when filtrates or extracts of cholera cultures were brought in contact with specific-immune sera, this method was not adopted for the purposes of routine laboratory diagnosis in view of the close correspondence found to exist between the results it gave and those
obtainable with the more expedient agglutination tests. Observations proving this rule have been made under various conditions, for instance, by Balteano & Lupu (1914) when using the sera of cholera-vaccinated individuals for parallel agglutination and precipitin tests, and by Damboviceanu et al. (1934) who applied both these methods to study the immunogenic properties of the residual antigen extracted from *V. cholerae* with the aid of trichloroacetic acid. It is also noteworthy that Gallut (1950), making precipitin tests with the glucolipidic extracts of *Br. suis* and cholera-immune sera on the one hand, with the corresponding extracts of cholera vibrios and various anti-brucella sera on the other, obtained results identical with those of analogous agglutination tests. However, Shrivastava & Seal (1937, see also Linton, Seal & Mitra, 1938) recorded that by making precipitation reactions with the vibrio polysaccharides isolated by them and various immune sera, it was possible to distinguish between cholera and El Tor vibrios which, though behaving identically in O agglutination tests, showed a different chemical constitution.

Shrivastava & Seal (1937) determined the precipitin reactions given by polysaccharides isolated from (1) a Group I Inaba strain of *V. cholerae* and (2) an Inaba variant belonging to the chemical Group VI of Linton and collaborators with the aid of immune sera representative of each of the six groups into which the vibrios were divisible on the basis of their chemical constitution. It was found that in the case of the typical Inaba strain, positive reactions were obtained only with Group I immune sera, while the Inaba variant reacted only with the two Group VI immune sera used. Three sera raised against El Tor vibrios falling into the chemical Groups IV or V failed to react with either of the two above-mentioned polysaccharides.

Continuing these studies with polysaccharides prepared from 23 vibrio strains belonging to different chemical groups, the haemolytic properties of which were not stated, Linton, Seal & Mitra (1938) reached the general conclusion that "precipitin reactions between the polysaccharides and antisera to the whole organisms indicate that in general the serology expresses the underlying chemical pattern of these organisms and indicates the same groups as the chemical analysis".

It also deserves attention that Gallut & Grabar (1943a) noted the presence of marked differences even among vibrios belonging to one and the same serological group, when quantitatively assessing with the aid of nitrogen determinations the precipitations which resulted from the action of fixed amounts of various immune sera upon variable amounts of the glucolipidic antigens of the organisms. They suggested that application of this method might be of value in defining the serological characteristics of the vibrios.

A profound study of the serological properties of *V. kadikoj*, a "haemotoxic" (haemolytic) vibrio belonging to the El Tor group, by Eisler &

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1 As quoted by Hetsch (1925), two Japanese observers, Fukuhara & Ota, noting that extracts of typical cholera stools gave specific precipitin reactions with cholera-immune sera, recommended the use of such tests for the purposes of practical laboratory diagnosis. However, as stated by Hetsch, no advantage has been taken of this theoretically interesting proposal. Recently Rainsford (1952) asserted once more "that a precipitin test could be devised which would indicate the presence of cholera 'O' antigen in stools of suspected cases", thus affording a rapid means for the presumptive diagnosis of the disease.
Kovacs (1926) showed that no relationship existed between the precipitinogen of this organism and its toxin, which was merely apt to become adsorbed to the flocculi produced by the action of the precipitating immune sera. A further important result of these studies was that Eisler & Kovacs were able to demonstrate the presence of two components of the precipitinogen, a thermolabile and coagulable one, which was adsorbable to animal charcoal, and a thermostable component resistant to boiling heat.

It is of importance to refer at the present juncture also to attempts to distinguish between cholera and cholera-like vibrios by precipitation tests with concentrated salt solutions. Liefmann (1913), following up casual observations made in this respect by Porges (1906) with ammonium sulfate, preferred for his work magnesium sulfate, used not only in varying concentrations for tube tests, but also for slide tests. For the latter purpose, loopfuls of the cultures to be examined were thoroughly mixed with drops of concentrated magnesium sulfate solution and also—to guard against wrong positives through spontaneous agglutination of the vibrios—with drops of normal saline placed on slides, results being read immediately.

Liefmann obtained in this manner precipitations in the case of 12 out of 14 cholera strains, whereas out of 9 cholera-like strains 8 gave entirely negative results. In tube tests 30 out of 40 cholera strains were well precipitated with 90% magnesium sulfate, 6 gave weaker and 4 negative results. Out of 20 cholera-like strains, only one reacted strongly, while the strain reacting positively in the slide tests produced a trace of precipitation; the other 18 strains gave negative results.

Greig (1913b), repeating such tests with a larger material, found that out of 176 cholera strains 164 were completely salted out, 12 only in traces, whereas out of 41 vibrio strains not agglutinable with cholera-immune serum only 6 showed a strongly positive reaction, 8 reacted weakly and 27 were not at all affected. Thus, as concluded by Greig, a close but not an absolute parallelism existed between these and agglutination tests. It follows that the method of salt precipitation does not furnish fully reliable results.

Complement-fixation tests

As can be gathered from the summaries of Köhlish (1910), Kolle & Schürmann (1912), and Hetsch (1912), the early application of complement-fixation tests for the purposes of cholera laboratory diagnosis (1906-07) soon led to considerable debates. Apart from the question whether with the aid of this method a differentiation could be made between cholera and cholera-like vibrios, it became at once a hotly contested point whether cholera and El Tor vibrios reacted identically or differently when tested in this manner with cholera-immune sera.

Advocating the latter opinion, Markl (1906) maintained that he had observed complete complement fixation when testing classical cholera vibrios with the aid of a cholera-immune serum, but only partial fixation
in analogous tests with El Tor strains—a difference which he ascribed to differences in the receptor apparatus of these two categories of strains.

Ruffer and Crendropoulo (see Ruffer, 1907), obtaining negative results in complement-fixation tests with El Tor vibrios, which reacted like the classical cholera strains in agglutination tests with cholera-immune serum, felt entitled to deny the specificity of the latter method.

There can be no doubt, however, that the technique used by these workers for their complement-fixation tests was unsatisfactory, particularly because they used suspensions of living organisms as antigens. As shown by Neufeld & Haendel (1907), it was impossible in this manner to obtain reliable results with haemolytic vibrios, because as a rule haemolysis became manifest after one hour in all tubes into which culture material of such organisms had been embodied. If, on the contrary, El Tor suspensions killed by half an hour's exposure to 70°C were used, results comparable to those with non-haemolytic strains could be obtained. Neufeld & Haendel established in this manner that "the El-Tor-vibrios also in complement-fixation tests with specific cholera sera react like true cholera bacilli".

Though a few subsequent workers again advocated views similar to those of Markl and of Ruffer, the validity of the findings of Neufeld & Haendel, which were soon confirmed by Besche & Kon (1909), is now generally accepted. Indeed, in view of the fact that no, or at least no marked, differences exist in the antigenic structure of cholera and El Tor vibrios respectively, it is impossible to assume that these two categories of organisms react differently in properly performed complement-fixation tests.

In addition to the above-described controversy, some of the early workers, for instance Schütze (1907), Neufeld & Haendel (1908), Baerthlein (1912), Michiels (1913), and Pottevin (1913a) expressed doubts as to the usefulness of complement-fixation tests for a differentiation of cholera and cholera-like strains.

However, the full specificity of the complement-fixation tests and the parallelism of their results with those obtainable with the aid of the agglutination method has been asserted by numerous other cholera workers, e.g., by Ballner & Reibmayr (1907), Bocchia (1911), Feldmann (1917), Kabeshima (1918a), and Mackie (1922), while Koshland & Burrows (1950) even came to the conclusion that the agglutinating and complement-fixing antibodies to the vibrio O antigen were closely similar, if not identical.

It has to be admitted that the practical value of complement-fixation tests in cholera-diagnostic work is limited, but this is due merely to the tediousness of the method and to the special technical requirements involved (Bocchia, 1911). Nevertheless, hand in hand with Pfeiffer's reaction or, in the case of avirulent strains, even in place of the latter method, complement-fixation tests were also recommended in 1907 by Weil. Some Japanese cholera workers, such as Uyeda (1924) and Fujimori (1928), laid stress upon the use of well-boiled antigens so as to abolish the inhibitory action of a supposed "impediment."
fixation tests might still prove of value in dealing with atypical cholera strains.

It is of interest to add that in place of live organisms or preferably of killed culture materials, some special antigens have been used for cholera-diagnostic complement-fixation tests. Thus Rondoni (1910) established that the nucleoproteid of *V. cholerae* was fully satisfactory in this respect and that, *vice versa*, subcutaneous injection of rabbits with this material produced sera containing complement-fixing antibodies. Kutscher & Schaefer (1916) proved through tests with rabbit immune sera that cholera vaccines formed suitable antigens for complement-fixation tests. Proposals have been made by a few workers, first apparently by Nedrigailoff (1909), to expedite cholera diagnostic work by directly using the patients' faeces as antigens in complement-fixation tests.

Nedrigailoff (1909) put fluid cholera stools into tall cylindrical glasses and after sedimentation used the supernatant as antigen. Complement-fixation tests performed instead with Berkefeld-candle filtrates of cholera stools gave almost completely negative results. Since the same held true if fresh broth cultures or suspensions of fresh agar cultures of *V. cholerae* were passed through the candles, whereas the filtrates of old cholera cultures proved to be suitable antigens in complement-fixation tests, Nedrigailoff postulated that—in contrast to the latter materials—cholera faeces contained no endotoxins.

Tokunaga (1911), carrying out complement-fixation tests with the faeces of cholera patients and cholera-immune sera, obtained positive results with 79% of his specimens. Faeces of cholera carriers gave, on the contrary, invariably negative results.

It was claimed by Amako & Kojima (1912) that, if the supernatant of typical cholera stools was used as antigen in complement-fixation tests, a diagnosis could be arrived at in 7-8 hours. Atypical stools containing only few vibrios had little or no antigenic value and it was necessary in such cases to use the upper layer of 6- to 10-hour-old peptone water cultures made from these stools in order to obtain good results in complement-fixation tests.

In view of the technical difficulties involved, it is not surprising to find that no large-scale advantage has been taken of the above-described method of cholera stool examination even in the past. At present, when highly specific media are available for a rapid direct isolation of *V. cholerae* from the stools, it is no longer possible to ascribe any practical value to it.

As stated by Svenson, some preliminary investigations by a Russian worker, Tuschinsky (1909), gave reason to hope that diagnostic advantage might be taken of complement-fixation tests with known cholera antigens and sera of patients with signs of choleraic disease. Amako & Kojima (1912), who further studied this possibility, used as antigens the combined washings of 3 or 4 agar-grown cholera strains. Making complement-fixation tests with human sera, they obtained positive results in 15 out of 34 mild cholera cases, in the case of 20 out of 28 patients with moderate to severe attacks of the disease and in 5 out of 17 cholera carriers. Complement-fixation tests with the sera of 2 patients with fulminant cholera and with those of 3 individuals with signs of cholera typhoid gave negative results. Commenting on these findings, Amako & Kojima stressed the necessity of using polyvalent
antigens of known activity, because—as has been generally acknowledged—
different cholera strains may give variously marked results in complement-
fixation tests.

In view of this evidence, it is undeniable that such tests might be used for
establishing the diagnosis of cholera currently or perhaps rather retro-
spectively, the more so because Yoshino (1922) established that complement-
fixation tests with known cholera antigens and normal human sera (as
well as with normal rabbit or horse sera) invariably gave negative results.
Still, for practical reasons it appears more expedient to use agglutination
tests, possibly also in vitro bactericidal tests, in preference to complement-
fixation tests for this purpose.

Balteano & Lupu (1914) found that complement-fixing antibodies
appeared in persons who had been vaccinated once against cholera 14 days
after the injection, and after 9 days in those who had in the meanwhile
received a second vaccine dose. In the former group the complement-
fixing property of the serum reached its maximum five days after it had
become manifest and disappeared within two months as against three
months in the case of the twice or thrice vaccinated.

Schoebl & Andaya (1925), who also performed complement-fixation
tests with the sera of cholera-vaccinated persons, used for this purpose
rather small vaccine doses, 28 of the individuals in question receiving one
dose of 500 million, 5 a single dose of 1000 million and 7 two doses of
500 and 1000 million respectively. Nevertheless, as revealed by tests made
one week or 12 days after vaccination, complement-fixing antibodies
became demonstrable practically always, the only exceptions being two
individuals who had received a single dose of 500 million and were tested
one week later. As shown by repeated tests, the complement-fixing anti-
bodies were apt to persist for 6-10 months. Both the length of persistence
and the titres reached seemed to depend to a higher degree upon the number
of the injections given than upon the amounts of vaccine administered.

**Phagocytosis tests**

Though profound studies by Neufeld & Hütte (1906, 1907) adduced
evidence that specific bacteriotropic substances, or, as they are usually
called, opsonins, rendering the vibrios liable to phagocytosis were present
in cholera-immune sera, and further investigations by Neufeld & Haendel
(1907) showed that El Tor vibrios were similarly influenced by the cholera
tropins, few attempts have been made to utilize methods based upon these
observations for the purposes of practical cholera diagnosis.

Schütze (1909), who seems to have been the first to devote attention
to such tests, evolved a technique of his own, described as follows:

An exudate rich in leucocytes was produced by injecting guinea-pigs intraperitoneally
with 10 ml of broth in which 2 g of aleuronat had been suspended, and puncturing the
peritoneal cavity of the animals 8 hours later. The exudate thus obtained was suspended
in normal saline in centrifuge tubes and twice washed in such saline with the aid of centrifugation, the resulting sediment, suspended in 4-5 ml of normal saline, being used for the tests.

To perform these, 1 ml of the leucocytic suspension was mixed in centrifuge tubes with equal amounts of (a) cholera-immune serum inactivated by heating for 20 minutes at 54°C, and (b) broth cultures of the vibrios to be tested. The mixtures were kept at 37°C for 10 minutes, then centrifuged for 1 hour. After the sediment had been twice washed in normal saline, it was used for the preparation of smears which, after heat-fixation, were stained for 3-5 minutes with alkaline methylene blue solution and then examined under the microscope in order to assess the degree of phagocytosis.

Carrying out such tests with a cholera and an El Tor strain as well as with three strains of cholera-like vibrios (Metchnikoff I and II, and Finkler-Prior), Schütze established that the former two vibrios were phagocytosed under the influence of cholera or El Tor immune sera to a considerably higher degree than the cholera-like vibrios, which were not at all ingested by the leucocytes when 1/50 dilutions of cholera-immune serum were used. Analogously, if a serum raised against \textit{V. metchnikovi} I was used, almost no phagocytosis of the heterologous strains resulted. However, no such differences were apparent, if instead a serum raised against \textit{V. metchnikovi} II was used in a dilution of 1/20. Schütze postulated, therefore, that the specificity of the vibrio opsonins was not absolute and that, though he had been able to distinguish with the aid of his method between cholera and cholera-like vibrios, this procedure alone should not be used for the purposes of differential diagnosis, agglutination tests, supplemented by Pfeiffer’s reaction, remaining the “main criteria” for this purpose.

Amako (1909), studying the opsonic properties of the sera obtained from 58 cholera patients or convalescents, came to the following conclusions:

1) According to my tests with cholera vibrios, normal sera and particularly cholera convalescent sera showed marked opsonic effects.

2) If fresh undiluted convalescent serum was used, the cholera vibrios were lysed extracellularly so that opsonic effects were not noticeable; if, however, serum dilutions were used, one could observe a clear [deutliche] opsonic action . . .

3) If the bacteriolytic property of the serum is too strong, so that one cannot recognize an opsonic action even if serum dilutions are used, one can observe an action after inactivation of the serum (through heating for 15 minutes at 60°C), because the cholera opsonins, like other immune-opsonins, are thermostable . . .” [Trans.]

As shown by Amako with the aid of numerous graphs, there existed in individual cases, as a rule, a parallelism between the agglutinatory, bacteriolytic, and opsonic properties of the sera.

It is of interest and of some practical importance to add that according to the investigations of Eisele, McCullough & Beal (1948) already referred to above, opsonophagocytic tests with brucellae, made according to the method recommended in the handbook on brucellosis in animals and in man of Huddleson (1943), were found to give positive results in 16 out of 20
cholera-vaccinated individuals. Marked reactions (phagocytosis by 80%-100% of the cells) were noted in about two-thirds of this group.

Allergic and skin tests

Shwartzman (1928) observed that if a preparatory intradermal injection of rabbits with a filtrate of _S. typhosa_ was followed 20-48 hours later by an intravenous administration of the same or a suitable heterologous bacterial filtrate, in the majority of the animals tested marked haemorrhagic lesions developed at the site of the preliminary injection, which were apt to undergo necrosis and ulceration.

As first established by Gratia & Linz (1931) and confirmed by Uyeda (1934) and Vassiliadis (1935c), this curious, though not, or at least not strictly, specific reaction, known under the name of the Shwartzman phenomenon, could also be produced by culture filtrates of _V. cholerae_.

Gratia & Linz (1931) found that this phenomenon could be produced not only in rabbits but also in guinea-pigs through a preliminary intradermal injection followed by a second injection into the jugular vein or into the heart. They also drew attention to the similarity of Shwartzman’s phenomenon with a reaction described by Sanarelli (1924a), according to whose observations a preparatory intravenous injection of rabbits with living cholera vibrios, followed 24 hours later by a second intravenous administration of either the homologous or a heterologous culture filtrate produced haemorrhagic reactions in the intestines, occasionally massive intraperitoneal haemorrhage, congestion of the genital organs apt to lead to abortion in pregnant animals, and sometimes immediate death.

Gratia & Linz tried, therefore, to produce Sanarelli’s reaction by the administration of cholera filtrates according to Shwartzman’s technique. Results were not uniform, some animals showing reactions neither in the skin nor in the intestine, others a typical Shwartzman reaction at the site of the intradermal injection, and some finally, instead of this, haemorrhagic intestinal reactions similar to those described by Sanarelli. One guinea-pig, which died 2 days after the second injection, though free from either skin or intestinal lesions, showed abundant blood clots in the peritoneal cavity and identical findings were made in another guinea-pig, which had been given a preliminary dose of 3 ml _V. cholerae_ filtrate intraperitoneally and had died a few hours after it had received a second dose intravenously.

The conclusion reached by Gratia & Linz was that there existed between the phenomena of Sanarelli and of Shwartzman “a close relationship or probably even an identity”.

Vassiliades (1935c), exploring whether Shwartzman’s phenomenon might be elicited with El Tor as well as with cholera vibrios, recorded the following results:

<table>
<thead>
<tr>
<th>Filtrate of</th>
<th>Number of animals</th>
<th>Dose for preparatory injection</th>
<th>Dose for intravenous injection</th>
<th>Results positive</th>
<th>Results negative</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. cholerae</em></td>
<td>3 rabbits</td>
<td>0.25 ml</td>
<td>1 ml per kg body-weight</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>4 guinea-pigs</td>
<td>0.11 ml</td>
<td>0.75-1.25 ml for 400-600 g</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Filtrates of</td>
<td>6 rabbits</td>
<td>0.25 ml</td>
<td>&quot;</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td><em>V. El Tor</em></td>
<td>8 guinea-pigs</td>
<td>0.10 ml</td>
<td>&quot;</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

It will be noted that (a) administration of _V. cholerae_ filtrates produced Shwartzman’s phenomenon in rabbits, but—in contrast to the findings of Gratia & Linz—not in guinea-pigs; and (b) on the contrary, filtrates of
two different El Tor strains failed to elicit this phenomenon. It would be of interest to establish with the aid of a larger material whether this difference between cholera and El Tor vibrios holds generally true.

Continuing investigations in this field, Raynal, Lieon & Fiessolle (1940) were able to produce local skin reactions in guinea-pigs, which had been immunized one month previously through intraperitoneal injection of live cholera vibrios, through intradermal administration of cholera antigens obtained with the aid of trichloracetic acid extraction. Normal guinea-pigs, receiving such antigens by the intradermal route, failed to react.

Kovacs (1932) reported that intracutaneous administration of the toxin of El Tor vibrios produced marked local reactions, consisting of infiltrations and hyperaemia or, if higher doses were used, even of necrosis, in the skin of rabbits, guinea-pigs and, as he showed on himself, also in the human skin. The appearance of these reactions could be inhibited by the simultaneous administration of sufficiently large doses of antitoxic sera and the reactions remained absent in guinea-pigs which had been immunized with the toxoid of the V. kadikoj, unless toxin amounts ten times exceeding the dosis necroticans were used.

Kovacs maintained that such intracutaneous tests could be used for a differentiation of El Tor and related vibrios from V. cholerae, because administration of 0.1 ml of the centrifugate of 6-day-old cholera broth cultures produced no skin reactions in a guinea-pig, whereas such reactions could be elicited with the centrifugates of El Tor and Kadikoj vibrios.

In contrast to these findings, Yu, Chen & Chen (1932) obtained positive skin reactions with toxic solutions prepared by (a) growing typical non-haemolytic cholera vibrios, the virulence of which had been enhanced by repeated guinea-pig passages, in buffered glucose-free broth; (b) centrifuging the culture fluid and filtering the supernatant through N Berkefeld candles. Reporting on their findings, Yu, Chen & Chen stated that

(a) three normal rabbits, intradermally injected with 0.1 ml of such a filtrate, showed skin reactions of considerable size;
(b) tests on 6 rabbits which had been immunized either with the toxic filtrate or with cholera vaccine, gave negative results;
(c) tests on human volunteers, who received 0.1 ml of the toxic filtrate intradermally into one forearm and, as a control, 0.1 ml of such a filtrate heated for 2 hours at 100°C, gave the following results:

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated</td>
<td>1</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td>Not vaccinated</td>
<td>60</td>
<td>3</td>
<td>63</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>30</td>
<td>91</td>
</tr>
</tbody>
</table>

* Positive reactions appeared within 6-12 hours, reaching a maximum between 20 and 24 hours and fading in 48 hours.

Yu, Chen & Chen suggested that their method might prove of value in testing individual susceptibility to cholera and also for checking the length
of the immunity produced by cholera vaccination. They noted in this connexion that the single vaccinated individual who gave a positive skin test had received cholera vaccine about three years before he had been examined.

The usefulness of skin tests for assessing the value of cholera vaccinations was also upheld by Brounst & Maroun (1949) who obtained positive reactions in 5 out of 10 thrice-vaccinated individuals tested by intradermal injection of 0.1-ml doses of the vaccine. Appearing after 48 hours, these reactions consisted of local congestion and oedema, sometimes accompanied by the appearance of a central necrotic zone or by a nodular infiltration.

Large-scale use of skin tests was made by Sabry (1950) during and after the 1947 cholera outbreak in Egypt. The antigen for these tests was prepared by (a) simultaneously growing three cholera strains in broth; (b) killing the organisms by exposure to 52°C for one hour; and (c) centrifugation, the lower more concentrated part of the centrifugate being used in a dilution of 1/300. Frankly positive results obtained with this antigen through intradermal injection consisted in the appearance of an oedematous papule surrounded by an erythematosus zone, the reaction becoming fully developed after 24 hours and then disappearing within 48 hours. In the case of mild reactions, no, or only an ill-defined, erythema became manifest round the papules.

Tests made in a cholera hospital on 13 patients, 9 convalescents, and 7 carriers produced only mild reactions, a result ascribed by Sabry to (a) a supposed state of allergy in these individuals, and (b) massive doses of sulfaguanidine which they had received. Sabry stated in the latter connexion that in the course of his further work he had succeeded in rendering the skin test negative by administering to a group of originally positive individuals 6 g of sulfaguanidine daily for 9 days.

Further results recorded by Sabry may be tabulated thus:

<table>
<thead>
<tr>
<th>Groups tested</th>
<th>Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
</tr>
<tr>
<td>5 stool-positive cholera carriers</td>
<td>5</td>
</tr>
<tr>
<td>37 vibrio-positive carriers</td>
<td>20</td>
</tr>
<tr>
<td>265 non-cholera patients</td>
<td>9</td>
</tr>
<tr>
<td>186 members of the hospital staff</td>
<td>32</td>
</tr>
</tbody>
</table>

Note. 31 individuals giving originally a negative skin test, remained negative when re-tested 7-30 days after they had received one dose of cholera vaccine.

Commenting upon further observations, Sabry stated that

"the percentage of the positive cases in the more recent experiments decreased among the domestic hospital staff as well as among ordinary cases, indicating that at least some of the carriers are on their way to recovery. As a decisive proof of the validity of this observation, 1001 cases were inoculated (i.e. skin-tested) during the period from 27-4-1948 to 6-7-1948 without the occurrence of one single positive reaction."
Sabry emphasized, therefore, the significance of the skin tests, maintaining that "the persistently positive cases represent the dangerous carriers responsible for the propagation of epidemics, who should be detected and kept under strict control". However, apart from the fact that most workers do not share Sabry's belief in a dangerous role played by carriers in the spread of cholera, it has to be kept in mind that in a majority of his observations he seems to have been unable to correlate positive skin tests with findings of *V. cholerae* in the stools of the individuals concerned.

### Natural Immunity

**Resistance and natural immunity**

As shown by ample experiences, and well illustrated by the classical observation of Macnamara (1876) that out of 19 persons drinking water from a vessel which had been accidentally polluted with fresh cholera excreta, only five actually contracted the infection, the ingestion of materials containing *V. cholerae* is by no means invariably followed by clinical manifestations of the infection. General agreement exists, however, that such a non-appearance of the disease is due, if not solely, at any rate mainly to an unspecific resistance to the infection instead of being the result of a specific natural immunity.

Various factors contribute to the unspecific resistance against cholera infection. As has been noted in the preceding chapter, some evidence has been adduced to show that the saliva of healthy persons exerts an antibacterial action on *V. cholerae* and might thus form a first line of defence against not too massive infection. Be this as it may, it is certain that the acidity normally prevailing in the stomach forms a potent barrier against the entry of cholera vibrios into the intestines where, owing to the presence of an alkaline reaction, conditions are favourable to the multiplication of the organisms. There can be no doubt, however, that even in the intestines unspecific defence mechanisms against cholera infection exist. The competition of the normally present bacterial flora is apt to exert an influence in this respect. Moreover, it is likely that a normal condition of the intestinal mucosa is capable of preventing an entrenchment of the invaders. Whether this defence mechanism is vested in the normally present mucous coating of the mucosa, as maintained, for instance, by Harvey (1929), or dependent upon the intactness of the epithelium itself (Romano, 1912), is difficult to decide. Probably both these factors play a role to a varying extent.

There can be no doubt, however, that the protection afforded to individuals in full health through the above-described means of an unspecific resistance to cholera is relative in degree. Kolle & Schürmann (1912), discussing this problem, pointed out with much reason that (*a*) like other acidophobe bacteria, cholera vibrios enclosed in copious amounts of food are apt to escape the action of the acid gastric juice, and (*b*) infected fluids,
particularly cold drinks, are apt rapidly to pass the stomach, particularly the empty stomach. Experimental observations supporting the latter contention have been noted already in Chapter 3. Kolle & Schürmann insisted also that even in normal persons the acidity of the gastric juice was subject to variation and could be low at times.

While thus even healthy persons are by no means invariably proof against an entry of *V. cholerae* into their system, ample observations have shown that individuals with a permanently low acidity of their gastric juice are particularly apt to fall victim to cholera infection. Thus it is a well-established fact that the disease is particularly rampant among individuals suffering from chronic gastritis due to the habitual abuse of alcoholic drinks. Sticker (1912) quoted in this connexion the observations made by Adams (1849) during an 1848-49 outbreak at Glasgow, according to which cholera killed 91 out of 100 drunkards as against 19 out of 100 abstemious persons. That temporary gastro-intestinal disturbances also favour cholera infection has been confirmed by observations on a greatly increased frequency of cholera admissions on the days immediately following Sundays or holidays, recorded during various outbreaks. Sticker, drawing attention to these records, also noted that according to several of the early observers the use of emetics and also that of even small doses of laxatives seemed to promote cholera infection.

The concept suggested by these observations, that, besides the normally prevailing acidity of the gastric juice, a normally present unspecific resistance of the mucous surface of the intestines prevents cholera infection, is supported by laboratory experiences. As will be fully discussed in the sixth chapter, several workers have succeeded in producing syndromes similar to, perhaps even identical with, human cholera in experimental animals ordinarily not amenable to oral infection with *V. cholerae*—in part by creating conditions analogous to those found to promote the appearance of cholera in man. Thus Pottevin & Violle (1913) recorded success in this direction by administering saline purgatives to monkeys before oral cholera infection, and Cantacuzène & Marie (1914) noted the appearance of a syndrome corresponding to that of human cholera in guinea-pigs, the resistance of whose intestine had been lowered through administration of podophyllin. Two guinea-pigs which had been dosed with this drug contracted the disease by mere contact with cholera-infected animals.

Most authorities are sceptical, and many frankly deny, that in addition to the well-documented presence of unspecific defence mechanisms a specific natural immunity against cholera infection exists. As far as it is permissible to adduce in this respect the evidence supplied by tests with the sera of normal, non-vaccinated subjects, it speaks against the presence of such a specific immunity, immune bodies being either altogether absent or demonstrable only at negligibly low titres.
Ample observations made in parenterally infected animals, though not directly applicable to the problem presently under review, are of interest in so far as they showed up a fundamental difference between an unspecific resistance and a specific immunity to cholera infection. It was found that previous administration of heterologous bacteria, such as *Chromobacterium prodigiosum*, *Proteus* and *Ps. pyocyanea* could protect guinea-pigs against intraperitoneal injection of lethal doses of *V. cholerae*. However, as shown by classical investigations of Pfeiffer & Issaeff (1894), the protection afforded in this manner was distinct from the specific immunity produced by the *V. cholerae* by its early appearance and rapid disappearance as well as by the failure of the non-specific organisms to produce cholera-immune sera.

*Naturally acquired immunity*

While it was maintained by some early observers that persons who had survived a cholera attack had become permanently immune against this infection, Koch (1884) held that this acquired immunity does not seem to persist for a long time because there is a sufficiency of examples to show that an individual who had been affected during one epidemic fell ill with cholera a second time during another outbreak; but one hears but rarely that somebody had been attacked twice during the same cholera epidemic. [Trans.]

However, Sticker (1912), emphasizing that several workers had observed cholera attacks in individuals who had recovered from the disease but some weeks previously (Spätrecidire), denied the development of a specific immunity against this infection and postulated that the rarity of second attacks was due merely to extrinsic causes, particularly the infrequency and short duration of the epidemics. Harvey (1929), discussing this problem, also adopted a cautious attitude, stating that the probabilities against an individual being in a position to contract a second attack of cholera must be great. This does not apply merely to his being in contact with cases of cholera, but to the likelihood of any individual's contracting infection even after the ingestion of the cholera vibrio.

Nevertheless, Harvey felt convinced that cholera attacks produce a naturally acquired immunity, but qualified this statement by adding that but little information exists as to the longer or shorter duration of that immunity.

As far as one can judge from the scanty evidence available on this point, it appears that cholera attacks, though not rendering the individuals concerned permanently immune, protect them against the infection for several years. This view was advocated for instance by Salimbeni (1915), who stated that the cured cholera patients are without any doubt immune to cholera for a longer or shorter time, because, though one knows of comparatively quite rare instances of individuals who had the disease two or even three times some years apart, as far as I know, no cases have been recorded of persons attacked by well-characterized cholera during one and the same epidemic. [Trans.]
Similarly it was recently stated by Maxcy (1951) that
"an attack of cholera does not necessarily confer protection against a subsequent attack. Nevertheless, second attacks within a period of a few years are uncommon."

A most interesting and important question arising in this connexion is whether, as considered probable by Harvey, the inhabitants of cholera-endemic areas, by suffering from slight and unnoticed attacks, become immune to the infection. It would be highly desirable to study this problem through large-scale investigations made in truly endemic areas with the aid of the immunological and experimental methods now available.

Dealing with the information then available on the presence of immune bodies in the sera of cholera patients and convalescents, particularly the experiences of Svenson (1909) referred to above, Hetsch made in 1912 the following statement:

"The experience that the agglutinin and bacteriolysin content of the blood in man and animals is considerably higher after cholera vaccination than after spontaneous cholera attacks and that nevertheless even after the slightest spontaneous attack the immunity is very considerably higher than after vaccination justifies the assumption that recovery from cholera produces a local immunity of the intestine, which is not produced to such a degree in animal experiments and through vaccination." [Trans.]

Plausible though this assumption is, it appears that Hetsch, again dealing with the problems of cholera immunology in 1928, laid no more stress upon a local immunity as contrasted to a systemic immunity against the infection. Be this as it may, it is certain that persons who have recovered from cholera can remain immune to the infection, even though no immune bodies are demonstrable in their sera.

**Active Induced Immunity**

*Introductory remarks*

The first attempt to confer protection against cholera through a method of active immunization was made by Ferrán (1885) during the epidemic rampant in Spain during 1884. Noting that guinea-pigs which had survived an injection of living cholera vibrios cultivated from faeces in broth were resistant to administration of further doses lethal to untreated animals, he applied this method to man. Ferrán made for this purpose initial injections of 8 drops of a broth culture of *V. cholerae*, to which bile had been added, and administered at intervals of 6-8 days two further doses of 0.5 ml each.

As summarized by Kolle (1896b) and by Voges (1896), it was soon shown by several workers that Ferrán worked with impure cultures containing only a minority of cholera vibrios besides numerous contaminating organisms. It is not surprising, therefore, that his method of vaccination not only gave no satisfactory results, but often produced severe, according
to some observers occasionally even fatal reactions. Modern writers are nevertheless unanimous in stating that Ferrán, though acting rather in­judiciously, deserves credit for having first demonstrated the possibility of an actively induced immunity against cholera.

It was the great merit of Camaleia (1888) to have first shown that it was possible to protect guinea-pigs against lethal doses of *V. cholerae* not only with the aid of living cultures, the virulence of which had been reduced, but also with growths killed by heating at 120°C. Curiously enough, the great practical importance of the latter observation was at first overlooked, Haffkine (1892b) recommending once more a method of cholera vaccination based upon the use of living organisms.

Following the scheme successfully used by Pasteur for rabies prev­ention, Haffkine used two cholera vaccines of different strength, administ­ering first a "weak virus" obtained through cultivation of cholera vibrios under continuous aeration at 39°C, and five days later a *virus fixe*, consisting of organisms the virulence of which had been exalted through repeated intraperitoneal passage directly from guinea-pig to guinea-pig. As sum­marized by Hetsch (1912), Haffkine used originally a suspension of a tenth part of a slant, prepared with boiled water, as a dose for adults, and gave to children 1/20th and to infants 1/100th part of a slant, but afterwards repeatedly changed these dosages, using for instance 1/12th and 1/8th of slants of the weak and the exalted virus respectively (Voges, 1896). Later on, in order to reduce the reactions and so to win the goodwill of the population, Haffkine often used lesser doses (1/20th of a slant).

The difficulties of using Haffkine's method of cholera vaccination on a large scale were tremendous. As he admitted himself (see Kolle, 1896a), it was a particularly heavy task to keep sufficient amounts of *virus fixe* available through continuous animal passages. In large-scale practice it also proved often impossible to administer second doses, so that only one-third of the 40 000 persons inoculated up to 1895 in India according to Haffkine's method received these. Nevertheless, some of the records published by him leave no room for doubt that his method was apt "to protect man against natural cholera infection" (Kolle, 1896b).

However, while admitting the value of Haffkine's method, Kolle (1896b) maintained that not only difficulties met with in manufacture but also considerations of a principal nature spoke against the use of live cholera vaccines. Believing that the toxins of *V. cholerae* were instrumental in conferring immunity, he considered it unnecessary to use strains of a particularly high virulence for vaccination, because according to the ob­servations of Dungern (1895) virulent cholera cultures were no more toxic than avirulent ones. It has to be noted in this connexion that, since according to the now-accepted views it is the antigenic structure and not the toxicity of the strains used for vaccine manufacture which is of decisive importance, the above contention of Kolle is no longer fully acceptable,
the use of virulent strains being advantageous at least in so far as these are bound to be smooth and therefore antigenically suitable. However, one must fully agree with Kolle's contention that the use of live instead of killed cholera vaccines offers no advantage in so far as, according to Haffkine's own observations, the organisms contained in his *virus fixe extraite* succumbed soon after injection. The action of live vaccines depended, therefore, not upon a survival of the organisms but upon the liberation of immunologically active substances contained in their bodies, as Kolle believed, of the toxins.

Far more important than these considerations were the results of comparative determinations of the bactericidal properties of the sera obtained from persons who had been vaccinated by Kolle (1896a, 1897) either three times according to Haffkine's method or once only with killed cholera vaccines, i.e., with suspensions of cholera cultures exposed (*a*) to heating at 56°C for one hour; or (*b*) to the action of chloroform vapours. Kolle established in this manner that the bactericidal titres of the sera derived from the individuals once injected with killed cholera vibrios were as high as or even higher than those of the persons thrice injected with Haffkine vaccine. This held true of the determinations made 10 days after the completion of vaccination as well as of those which could be made one year or even longer afterwards. Kolle (1896a) summarized, therefore, that "since an equal effect is obtained, it is better to use *sterile* cholera vaccines than vaccines containing *living* vibrios, because the manufacture of the latter (production of the cultures) is difficult as well as dangerous. Further, the pain produced by the injections deters many from submitting to a second inoculation. We know through my investigation that one injection of a somewhat larger dose... produces the same effect as multiple inoculations." [Trans.]

It is somewhat surprising to find that in spite of these observations of Kolle a few subsequent observers again advocated the parenteral use of live cholera vaccines. Thus Nicolle, Conor & Conseil (1912) used living virulent cholera vibrios for intravenous injections.

As summarized by Hetsch (1928), these workers prepared their vaccines by subjecting suspensions of 20-hour-old agar cultures to centrifugation followed by repeated washing and final resuspension of the sediment, one drop of the fluid thus obtained being adjusted to contain 4 million of *V. cholerae*. This amount, diluted in 50 ml of normal saline, was used as the initial dose for adults and this was followed 10-15 days later by administration of a dose six times stronger.

Nicolle and co-authors stated that the 36 persons thus vaccinated showed no serious reactions, particularly no diarrhoea. The application of the vaccine led to the abundant formation of antibodies in the sera of these

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1 The method of intravenous administration of cholera or mixed vaccines was also recommended by Quarelli (1917). He found that this method of vaccination, while causing but mild reactions, led to a more rapid and persistent production of antibodies than the subcutaneous method.
individuals and three of them remained healthy when afterwards given virulent cholera vibrios per os.

Castellani (1913) stated that he had prepared a live attenuated cholera vaccine for subcutaneous injection by heating 48-hour-old cultures for one hour at 48°C or 45°C. However, these vaccines produced much more severe local and general reactions than killed cholera vaccine and a further disadvantage was that the attenuated vaccines had to be used soon after preparation, because, even if heating at only 45°C had been resorted to, the organisms died within two months. One may truly say, therefore, that there is nothing to recommend the use of Castellani’s live vaccine in actual practice. The method of Nicolle and colleagues seems to be capable of producing a solid immunity, but its large-scale use would be fraught with great difficulties and even some danger. It is not surprising, therefore, that —apart from some attempts to practice oral vaccination against cholera—parenteral administration of killed vaccines or, to a lesser extent, of extracts prepared from *V. cholerae* in various ways, has been adopted as the standard practice for large-scale vaccination campaigns. The various methods used for this purpose, and also the problem of oral vaccination, will now be dealt with seriatim.

**Agar-grown killed vaccines**

Agar-grown killed vaccines have been continuously used for the purposes of cholera control since their recommendation by Kolle in 1896, first on a large scale during the 1902 cholera epidemic in Japan (Murata, 1904; Takano, Ohtsubo & Inouye, 1926).

While it would be redundant to enter into a detailed description of the technique of preparing agar-grown vaccines, which is set forth in the textbooks on bacteriology and laboratory methods, it is of importance to discuss the following special problems of their manufacture, standardization, administration, and storage.

1. **Choice of strains.** Dealing with the problem of selecting strains suitable for the manufacture of cholera vaccines, Hetsch (1912) noted that some of the early workers had insisted upon the necessity of choosing highly virulent strains, while others reached a contrary opinion, pointing out that part of the avirulent strains had equally good antigenic properties. Agreeing with the latter view, Hetsch emphasized that the immunizing properties of cholera strains did not run parallel with their virulence and that it was essential, therefore, to select those strains which showed a marked antibody formation in preliminary experiments. However, as will be stated below, some workers have recently again laid stress upon a high virulence of the strains used for the preparation of cholera vaccines. As noted before, it is certain that the use of freshly isolated (and, therefore, presumably virulent) strains is of great importance in so far as these are unlikely to have undergone a loss in antigenic properties through roughening.
Though the phenomena of dissociation were still unknown at the time, it can be gathered from the summary of Hetsch (1928) that most workers engaged in the manufacture of cholera vaccines during the First World War insisted upon the use of freshly isolated strains. It was usually recommended to select several of these for the preparation of polyvalent vaccines.

Schwarz (1919), one of the workers quoted by Hetsch, aptly distinguished between vaccines manufactured from strains isolated locally during a cholera outbreak (epidemieeigene Impfstoffe) and those prepared from strains of a heterologous origin (epidemiefremde Impfstoffe)—either polyvalent vaccines or vaccines made from single selected strains. The criteria for choosing such specially suitable strains, established by tests with individually prepared vaccines, were according to Babes (1914) (a) absence of a severe reaction after vaccination, and (b) marked immunizing properties, as shown through tests in guinea-pigs injected simultaneously with dilutions of the sera of persons who had been given doses of the vaccines in question, and lethal doses of V. cholerae.

In the experience of Schwarz it was of importance to use vaccines prepared locally during the epidemic to be dealt with. He noted in this connexion that individuals who contracted infection even though they had been injected with such vaccines, had slight attacks or merely became carriers of V. cholerae, whereas usually severe forms of the disease were observed in persons who fell ill with cholera though they had received injections of heterologous vaccines.

The necessity of using fully smooth cholera strains for vaccine manufacture was stressed by Steward (1933). He noted that in actual practice during the off-seasons eight subcultures were made from a recently isolated strain, which were kept in the refrigerator and successively used to prepare vaccines. During the epidemic seasons freshly isolated smooth strains were used for this purpose and were frequently replaced by strains of the same character. Thus, as Stewart stressed, the old method of preparing cholera vaccines from stock strains had been given up. Many of the subcultures made from the latter showed roughness.

Dealing again with the problem of selecting cholera strains for vaccine manufacture, Taylor, Ahuja & Singh (1936) stated that:

“...The maximum degree of protection in animals against infection with strains of the prevailing serological type is obtained by the use of vaccines prepared from strains which show both ‘H’ and ‘O’ agglutination with a serum of the Japanese ‘original’ type and which also show the chemical structure (Linton’s groups I and II) characteristic of the majority of agglutinable vibrio strains isolated from cases of cholera in India. Agglutinable strains from carriers and agglutinable variants produced from strains of origin other than cholera cases give a lower degree of protection.”

1 The statement made in the text of Stewart’s article that these subcultures were kept in the incubator is obviously due to an error in translation, because he noted that, in contrast to cholera cultures kept in the refrigerator, those left at room temperature or in the incubator had a great tendency to become rough.
Quoting recommendations made by the Cholera Advisory Committee of the Indian Research Fund Association, Taylor (1941) stated more specifically that:

"The strains ordinarily used in manufacture of (cholera) vaccine should show the following characters:

(a) Typical smooth translucent colony appearance.
(b) Stable in salt solution.
(c) Serological characters of O group I (Gardner and Venkatraman) sub-type Inaba and should agglutinate to titre with a serum prepared against the dried O Inaba antigen issued from the Standards Laboratory, Oxford.
(d) Producing acid from mannose and saccharose but not from arabinose.
(e) Non-haemolytic."

Taylor added that in the opinion of the Cholera Advisory Committee there was no evidence to show whether the use of multiple strains for cholera vaccine manufacture was necessary or not. However, since in certain areas strains of the Ogawa subtype had been isolated from a considerable number of cholera cases during epidemics, it was in the opinion of the Committee "for consideration whether strains for the Ogawa subtype should be incorporated in the vaccine and this is the practice in the Madras Presidency".

Yu (1938, 1942) laid emphasis upon selecting fully virulent strains for the manufacture of cholera vaccines.

He recorded in this connexion the following results obtained when (a) injecting groups of mice at intervals of 3 days with 2 doses of heat-killed cholera vaccines (1000 and 2000 million respectively), which had been prepared individually from 6 strains varying in virulence, and (b) challenging the animals 20 days afterwards with 3 MLD of one of these strains:

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Virulence of organisms</th>
<th>Number of mice tested</th>
<th>Survived</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>++++</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>(b)</td>
<td>++++</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>(c)</td>
<td>+++++</td>
<td>40</td>
<td>31</td>
</tr>
<tr>
<td>(d)</td>
<td>+</td>
<td>47</td>
<td>18</td>
</tr>
<tr>
<td>(e)</td>
<td>-</td>
<td>48</td>
<td>18</td>
</tr>
<tr>
<td>(f)</td>
<td>-</td>
<td>20</td>
<td>8</td>
</tr>
</tbody>
</table>

Yu added that stock cholera cultures were not suitable for vaccine manufacture because they were not perfectly smooth. They could be rendered negative to Millon tests by repeated mouse-passages but these led to only a slight rise of the virulence of the strains.

During the following years considerable attention was paid to the question whether a cross-protection existed between the Inaba and Ogawa subtypes of *V. cholerae* and whether, consequently, monovalent cholera vaccines or vaccines consisting of a mixture of equal parts of vaccines prepared from Inaba and Ogawa strains respectively should be issued.

Ranta & Dolman (1944) recorded in this connexion the following observations:
"... Each of 50 mice was inoculated with the prescribed two doses of vaccine prepared from a single Inaba-type strain. A fortnight after the final dose, half of this vaccinated group was challenged with 10 m.l.d. of the Inaba-type strain, and half received 10 m.l.d. of an Ogawa-type strain. A similar number of mice, inoculated with 2 doses of Ogawa-type vaccine were divided into two groups, which were challenged with 10 m.l.d. of Inaba- and Ogawa-type vibrios respectively. All vaccinated mice survived."

Though in the opinion of Ranta & Dolman these findings did not necessarily imply that the type-specific O antigens played a part in mouse protection, they pointed nevertheless to the existence of a cross-protection between the two subtypes of *V. cholerae*.

This postulation was fully supported by Burrows et al. (1947), who concluded from large-scale active and passive immunization experiments that there existed "complete cross protection between vibrio types".

For a further study of this problem, Ahuja & Singh (1948) injected guinea-pigs subcutaneously with two doses of cholera vaccines prepared respectively from strains of the two subtypes or with a mixed Inaba-Ogawa vaccine and challenged the animals 10 days after the second injection intraperitoneally with mucinized suspensions of live cholera vibrios of either the Inaba or the Ogawa subtype. Tabulated, the results of these tests were as follows:

| Vaccine used | Number of guinea-pigs immunized | Type of challenge strain | Survival up to 36 hours (\%)
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Inaba</td>
<td>15</td>
<td>Ogawa</td>
<td>14 93.0</td>
</tr>
<tr>
<td>Inaba</td>
<td>15</td>
<td>Inaba</td>
<td>14 93.0</td>
</tr>
<tr>
<td>Ogawa</td>
<td>14</td>
<td>Ogawa</td>
<td>14 100.0</td>
</tr>
<tr>
<td>Ogawa</td>
<td>15</td>
<td>Inaba</td>
<td>13 89.0</td>
</tr>
<tr>
<td>Inaba + Ogawa</td>
<td>15</td>
<td>Ogawa</td>
<td>15 100.0</td>
</tr>
<tr>
<td>Inaba + Ogawa</td>
<td>15</td>
<td>Inaba</td>
<td>15 100.0</td>
</tr>
</tbody>
</table>

*Note: None of the 30 non-vaccinated controls challenged with either the Inaba or Ogawa type vibrios survived.*

Though considering that the results obtained with the mixed vaccine were not significantly different from those obtained with the two monovalent vaccines, Ahuja & Singh concluded that

"On the basis of these findings the use of both sub-types of *V. cholerae* for the preparation of prophylactic cholera vaccine would be more satisfactory than the use of either an Inaba or an Ogawa sub-type alone."

Dealing not only with the problem presently under review but with the selection of strains for the purpose of cholera vaccine manufacture in general, Pandit (1948) made the following important statement:

"In view of the evidence ... regarding the prevalence of subtypes of vibrios in India, the vaccines used in the country are prepared from both the types of vibrios, particularly those isolated from fatal cases of cholera. It is customary in most laboratories to replace the strains used by new ones as they are isolated. Pending further information on the question of virulence of vibrios, this procedure was considered to be the most suitable for adoption in the manufacture of cholera vaccines. However, it would seem that with
the development of the technique for the measurement of antigenicity of vibrio strains, it should be possible to select such strains only for vaccine production as show a sufficient high degree of antigenic potency. Recently Ranta & Dolman (1944) and subsequently Burrows and his collaborators (1947, p. 157) obtained evidence to show that practically complete cross protection exists between the two sub-types of cholera vibrios. However, recent observations by Venkatraman in the King Institute tend to show that this may not always be the case, particularly if minimal quantities of antigens are used for protection."

As will be discussed below, the conclusion tentatively reached by Venkatraman was vigorously supported by Sokhey & Habbu (1950b), who denied that a cross-protection existed between the Inaba and Ogawa sub-types.

Under these circumstances it seems indicated for the present to use both Inaba and Ogawa strains for the manufacture of cholera vaccines destined for wide distribution. Though, as shown by observations like those of Burrows et al. (1947) and of Sokhey & Habbu (1950b), the virulence of cholera cultures may be preserved for prolonged periods through freeze-drying (lyophilization), it is certainly best to replace the strains used for vaccine preparation by recently isolated ones whenever possible.

(2) Cultivation methods. While, generally speaking, the high-quality agar media available for diagnostic work are also utilized for the manufacture of cholera vaccines, some workers recommended for the sake of economy cheaper media, for instance, one prepared with 3% yeast instead of with meat or meat extracts and peptone (Fischer, Bitter & Wagner, 1915). However, Ungermann (1917), giving a systematic description of the methods of cholera and typhoid vaccine manufacture in the Berlin Gesundheitsamt, warned against the use of cheap substitutes, particularly prefabricated meat extracts which, because possibly made from meat of doubtful freshness, were apt to contain products of protein decomposition and thus to cause untoward reactions in the vaccinated. Since, moreover, media made with fresh beef gave a more abundant growth than those prepared with horse meat, the former alone were used for vaccine manufacture in the Gesundheitsamt. However, as shown by the excellent quality of the cholera vaccine made in the Kasauli Institute in India, in countries where the use of beef for media preparation is out of the question, mutton digests are apt to prove equally advantageous.

While, in general, Roux bottles or similar containers or, as recommended by Ungermann, large covered glass dishes (diameter 21 cm) are used in vaccine manufacture, Fischer and collaborators (1915) advocated the use of tin dishes. They admitted, however, that these became rusty after they had been used several times.

Ungermann claimed that more abundant yields were obtained when, instead of suspensions made directly from agar subcultures, ad hoc subcultures made from these in broth (Bouillonvorkulturen) were used for seeding the flasks or dishes. He adduced as explanation that in agar cultures of V. cholerae even after a growth for only 24 hours a part of the
organisms were no longer viable, whereas in broth the overwhelming majority of the vibrios were still in the stage of multiplication at that time.

As generally agreed, an incubation at 37°C for 20-24 hours is suitable for the mass production of *V. cholerae* in the course of vaccine manufacture.

(3) Killing methods. The first, and until rather recently the most amply used, method for killing the vibrios in the course of cholera vaccine manufacture was to expose the organisms to heat. As noted above, Gamaleia (1888) resorted in his pioneer work to a temperature of 120°C. Fairbrother (1928) established that the substance of *V. cholerae* which on inoculation gave rise to protection in animals was heat-stable, withstanding an exposure to 100°C for one hour. Uyeda (1922) claimed that a "koktoantigen," i.e., the supernatant obtained through centrifugation of *V. cholerae* suspensions which had been boiled for 15-30 minutes, was the best cholera vaccine.

Though it is not possible to share this view, it is noteworthy that according to the observations of Taylor (1936), Burrows et al. (1947) and Singer (1948b) prolonged boiling exerted no untoward influence on the immunogenic power of cholera antigens.

Notwithstanding these observations, the general tendency has been to use for heat-killing the organisms in actual cholera vaccine manufacture even lower temperatures than that of 56°C recommended by Kolle in 1896. Haffkine (1913), who in about 1911 began to use a killed cholera vaccine, resorted for its preparation to an exposure of the vibrio suspensions to only 50°C for a few minutes, but followed this procedure by the addition of 0.5% phenol. Generally speaking, however, temperatures of 53°C to 54°C have been applied for the period of one hour. If carefully implemented, this method gives fully reliable results, particularly if, according to the generally adopted practice, phenol to a final concentration of 0.5% is added as soon as initial samples have been withdrawn to test the sterility of the brews.

As summarized by Hetsch (1928) and Harvey (1929), methods to effect sterilization of agar-grown vaccines through the addition of antiseptics instead of through the application of heat have been recommended by several workers, the substances used being—besides 0.5% phenol—chloroform, ether, formol, glycercol, hydrochloric acid, and quinine. Singer, Wei & Hoa (1948b) found an alcohol-killed vaccine as satisfactory as those killed by heat or other methods.

Tentative use of 0.5% phenol was made already early in his work by Haffkine with the idea of abating the virulence of the cholera strains used for the preparation of his "weak virus." However, as noted by Hetsch (1912), it was soon shown by some other workers that this chemical exerted a sterilizing instead of an attenuating effect. Large-scale advantage of the
method of sterilizing cholera vaccines with phenol alone was afterwards taken by many workers, e.g., those in Japan (see Takano and colleagues, 1926), and this procedure is still widely used. As quoted by Taylor (1941), the Cholera Advisory Committee of the Indian Research Fund Association recommended in this respect that:

"The vibrios should be killed by the addition of 1 per cent phenol to the suspension without the application of heat. The phenol should be reduced to 0.5% in the vaccine finally issued."

With the exception of formol which, as will be noted below, has been used to a considerable extent for the sterilization not only of agar-grown but also of other types of cholera vaccines, the other chemicals enumerated above have not been used routinely in the manufacture of such vaccines.

(4) Standardization methods. Introducing the method of cholera vaccination with agar-grown vaccines, Kolle (1897) recommended that these ought to contain one "normal" loop or 2 milligrams of fresh culture mass per millilitre. To ensure this standard, or that of 2 loops (4 mg) per ml required in Germany at the time of the First World War (see Ditthorn & Loewenthal, 1915; Fischer, Bitter & Wagner, 1915), some workers merely computed the number of loops by determining the surface area of the media used for cultivation, assuming that one agar slant yielded 10 loops of culture mass, a Petri dish of a diameter of 8.8 cm, 66 loops, etc. (Soltmann, 1915). However, this rather crude method of standardization was criticized by other workers, for instance by Ungermann (1917), who pointed out that (a) successively prepared media were apt to give different yields, and (b) more important still, the abundance of growth depended not merely upon the surface area of the media, but also upon their mass and differed, therefore, according to the thickness of the agar layers. For these reasons the above-described method of standardization has been given up in favour of determinations of the bacterial contents of vaccines with the aid of gravimetric or counting procedures, or by opacity tests.

General agreement has been reached that the method of weighing in the course of vaccine manufacture the moist culture masses does not yield exact results, mainly because their weight depends upon a large and variable water content rather than upon their bacterial content. As shown by careful observations such as those of Brown (1914, 1919) and of Ungermann (1917), determinations of the dry weight of the bacterial masses give fully reliable results, but this method is not only rather tedious but of limited value in so far as, whether rightly or wrongly, standard values of bacterial vaccines are usually given not in terms of weight but in numbers of the organisms per ml, which have to be determined with the aid of counting methods.

As far as the preparation of cholera vaccines is concerned, only two of the latter methods have been used on a large scale, namely, that introduced
by Wright in 1902 and haemacytometer counts recommended about the same time (see summary by Soltmann, 1915). As is generally known, the principle of the former method is to mix equal volumes of the bacterial suspensions to be tested and of fresh human blood in a capillary tube, to prepare stained smears from this mixture and to compare the number of bacteria present with that of the blood corpuscles. Since it may be taken that 5 million of the latter are present per ml, this standard value can be used easily to compute the number of organisms per millilitre.

Though the present writer for one cannot agree with the assertion sometimes made that Wright's method gives inconsistent results, it is generally admitted that the number of organisms elicited with its aid is lower than that actually contained in the bacterial suspensions under test. There can be little doubt, therefore, that haemacytometer counts, which give exact values, are preferable for vaccine standardization but unfortunately, simple as the implementation of this method seems at first glance, it is fraught with considerable technical difficulties and thus reliable only in the hands of experienced workers. It is under these circumstances of great importance that, as shown by ample experiences, results approaching or even equalling in exactness those obtained by dry weight determinations or properly made counts may be obtained with the aid of opacity tests.

No doubt it would be simplest to carry out the latter by comparing the opacity of the bacterial suspensions under examination with that of previously prepared vaccines. However, though used by some workers, in the experience of most observers this method is as unreliable as it is expedient, because bacterial vaccines, quite particularly cholera vaccines, are apt to undergo a process of autolysis, thus changing in aspect and density. It is necessary, therefore, to carry out the opacity tests with the aid of standard suspensions of a non-bacterial nature, preferably those of a stable character which can be used on successive occasions.

Various substances have been suggested or actually used for this purpose. Ungermann (1917) noted in this respect that emulsions of lecithin in water, though formerly recommended for opacity tests, possessed in higher concentrations a colour of their own which interfered with their suitability for the standardization of cholera vaccines. He recommended, therefore, the use of alcohol dilutions of a 10% solution of dry Canada balsam in benzol. A suspension corresponding in opacity to that of cholera vaccines of the officially prescribed strength could be obtained by mixing 0.6 ml of the balsam-benzol solution (which was stable) with 9.4 ml of absolute alcohol. Results with this method were so satisfactory that it alone was used in the Berlin Gesundheitsamt for the standardization of cholera and typhoid vaccines.

As shown by some other workers, particularly through the exhaustive studies of Brown summarized in his publication in 1919, reliable results in the standardization of bacterial vaccines could be obtained through
opacity tests made with the aid of standard barium sulfate suspensions. According to Brown, these quite stable suspensions could be prepared as follows:

"A strong solution of barium chloride is made and to this is added an excess of sulphuric acid. The mixture is then boiled and the precipitate is poured on to a filter paper and is washed with tap water until the filtrate is neutral to litmus paper.

"The barium sulphate is then dried and thoroughly roasted. When cool a portion is accurately weighed and placed in a perfectly clean mortar. The powder is finely ground and the requisite amount of 1 per cent aqueous solution of sodium citrate is gradually added. From this 1 per cent suspension of barium sulphate an 8-fold dilution is made and similarly the other dilutions, the sodium citrate being used throughout as the diluent fluid."

A set of 10 tubes was thus prepared, the first containing the above-mentioned eightfold dilution, the second 9 volumes of the same dilution and 1 volume of citrate solution or, in comparison to the first tube, 90% barium sulfate and so on, the last tube containing 1 volume of the original barium sulfate suspension and 9 volumes of sodium citrate solution.

Brown furnished the following figures showing the relationship of opacity to the weight of dried bacterial substance, expressed in milligrams per ml of *V. cholerae* suspensions:

<table>
<thead>
<tr>
<th>Percentage of BaSO₄ suspensions*</th>
<th>Serial number of opacity, tube</th>
<th>Weight of vibrios (mg per ml)</th>
<th>Numerical equivalent of vibrios (millions) **</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.0</td>
<td>10</td>
<td>2.22</td>
<td>10925</td>
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<tr>
<td>90.0</td>
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<td>1</td>
<td>0.22</td>
<td>1093</td>
</tr>
</tbody>
</table>

* As defined in text
** As established with haemacytometer tests by Cunningham & Timothy (1924)

As stated by Gardner (1931), bacterial vaccines may be roughly standardized as follows:

"A barium sulphate suspension is made by mixing equal parts of M/100 H₂SO₄ and M/100 BaCl₂. This is shaken and distributed into a series of 6 by 5/8 in. test tubes covering the maximum variation in diameter of the stock in use. They are then sealed. The measured bacterial suspension is diluted in a test tube until it is equal in opacity to the standard in a tube of the same diameter. The bulk is diluted to the same extent or retained as a known multiple of the standard. Equal opacity is judged by viewing a small luminous flame through the tubes. The opacity will be equal in each if the image is obscured in each at the same distance from the flame."

Joetten (1917), dissatisfied with the exactness of all the above-mentioned methods, tested cholera and typhoid vaccines with the aid of complement-fixation tests, using the vaccines as antigens and a bacteriolytic cholera-immune serum as amboceptor. Though he found that vaccines which
appeared to be of uniform value gave identical results in such tests, the
implementation of this method in the course of mass vaccine production
would be fraught with considerable difficulties. The same holds true of the
important proposal of Gallut (1949c) to standardize cholera vaccines by
determining the weight of the O antigen extracted with the aid of trichlor­
acetic acid. Gallut maintained in this connexion that only strains with an
adequate yield of O antigen, equalling at least 5% of the total dry weight
of the organisms, should be used for cholera vaccine manufacture.

It is important to add that an international reference preparation for
opacity tests has recently been introduced by the World Health Organization
on the recommendation of its Expert Committee on Biological
Standardization; this preparation is held for distribution to national labor­
atories for biological standards by the Statens Seruminstitut, Copenhagen,
Denmark. As stated in a description of the manufacture and properties
of this preparation by Maaløe (1955), the material constituting it is a
suspension in distilled water of small particles of Pyrex glass, as used
earlier as a working standard for the characterization of pertussis vaccines
and suspensions of challenge bacteria in the Bethesda laboratory of the US
Public Health Service. Noting that the standard preparation is adjusted to
correspond in opacity to a pertussis vaccine with 10 000 million organisms
per ml, Maaløe emphasized that

"Such translation of opacity into numbers of organisms per ml should, however,
not be attempted generally; it should be stressed that the growth conditions and the
subsequent treatment of the organisms making up a vaccine may greatly influence the
opacity of the vaccine taken as a function of the number of organisms per ml (this is most
pronounced in the case of cholera vaccines). If it is desired, nevertheless, to translate
opacity units into number of organisms per ml, this number should be determined very
carefully on a suspension of bacteria which have been treated in a specified manner.
The conversion factor obtained for this batch should be used to translate opacity units
into number of bacteria per ml only when dealing with suspensions of bacteria grown
and subsequently treated in this particular manner."

(5) Dosages. Kolle (1897), finding that a single administration of his
vaccine, standardized to contain 2 mg of culture mass per ml, produced a
satisfactory antibody response in a group of volunteers, maintained that
vaccination with such single doses was sufficient for the purposes of cholera
control. As noted before, actual use of this method was first made during
the 1902 epidemic in Japan. Reporting on this work, Murata (1904) stated
that the dosage recommended by Kolle, though giving good results in
general, was not invariably sufficient, a number of the vaccinated con­
tracting the infection. He soon resorted, therefore, to single administrations
of 1-ml doses of a vaccine of double strength (4 mg of culture mass per ml)
and found that none of the persons thus protected contracted cholera.

As has been noted above, a standard of 4 mg culture mass per ml of
cholera vaccine was also made obligatory in Germany, but this vaccine
was administered to the armed forces during the First World War in two
doses, usually of 0.5 ml and 1 ml, the second injection being given after an interval of 5-7 days. According to Takano and co-authors (1926), a two-dose system of cholera vaccination was also soon adopted in Japan where, however, 1 ml and 2 ml respectively of a heat-killed and phenolized vaccine containing only 2 mg of the vibrios per ml were administered.

While the principle that, in order to confer a satisfactory degree of protection against cholera, a two-dose system of vaccination should be adopted has been generally accepted, in the experience of many field workers it is often impossible to act accordingly in large-scale vaccination campaigns. This situation has been well described by Russell (1935) who stated that

"The Cholera Commission of the Office international has recently reiterated its view that 'while vaccination by a single injection is of some value and can be employed in circumstances in which it is the only practicable method, vaccination by two injections should remain the method of choice.' In countries like India, however, where the number of inoculations to be done urgently, may run into many thousands, it is usually impossible to give more than a single inoculation and the common practice is to give a single dose of 1 cc."

It is clear that in order to obtain the best possible results with the system of single-dose vaccination, which had to be adopted for large-scale campaigns not only in India but also in other countries, for instance in China, it is essential to use a high-standard vaccine so as to avoid the administration of too low dosages—a practice unfortunately not really resorted to—or to obviate the considerable drawbacks of injecting amounts in excess of 1 ml. This desideratum has been satisfactorily fulfilled in India, where a standard of 8000 million of V. cholerae per ml has been adopted. In some other countries, however, cholera vaccines of lesser strength were used, e.g., in China often one of only 2000 million of organisms per ml, administered as a rule in amounts of 1 ml. It was only after Dzen & Yu (1936) had demonstrated the inadequacy of this method through large-scale guinea-pig experiments, that better counsel prevailed, and Robertson & Pollitzer (1939), working under the auspices of the League of Nations, were able to introduce a vaccine with a vibrio content of 6000 million per ml—a standard afterwards widely adopted in China. However, while single-dose administration of this vaccine gave satisfactory results, the present writer wishes to emphasize once more that this system of cholera vaccination was adopted merely out of necessity and that it ought to be replaced whenever possible by the administration of two vaccine doses.

(6) Keeping qualities. As confirmed by numerous observations, for instance by the special studies of Ungermann (1917), killed vaccines, quite particularly cholera vaccines, soon begin to undergo a progressive process of disintegration of the bacterial cells.

As maintained by Lange (1922), this process, though often designated as autolysis, is really one of cytolysis and thus fundamentally different from autolysis in the strict sense, due in Lange’s opinion to a decomposition of the bacterial protein through the
action of ferments, which had not been destroyed during the process of killing the vaccines by moderate heat nor by the subsequent addition of 0.5% phenol and were thus able to produce a progressive and thorough reduction of the antigenic properties of the vaccines. Since it was inadvisable to destroy these ferments by the application of higher temperatures, Lange recommended that formol ought to be used to kill the organisms and to destroy the ferments at the same time. He evidently assumed that autolysis in the strict sense frequently took place in cholera vaccines killed by other methods, but this is not supported by the experiences of other workers quoted below.

In contrast to the contentions of Lange, Dold (1925) maintained that less “autolysis” took place in heat-killed vaccines than in those sterilized by other methods, both because a kind of protein coagulation took place at temperatures of about 60°C and because such temperatures damaged the autolytic ferments.

A further study of this problem was made by Gohar & Makkawi (1948). Finding that some of the numerous brands of vaccine used for combating the 1947 cholera epidemic in Egypt after a few weeks’ storage no longer conformed to the prescribed standard of 8000 million per ml, these two workers compared the lysability of cholera vaccines killed and preserved in different ways, making for several days daily counts with a photoelectric colorimeter. It was thus established

“that heat-killed vaccines were by far the most stable, apparently because of the adequate coagulation of protein by the heat. Formalin-killed vaccines were also stable but they keep best when preserved with merthiolate instead of phenol. The phenol-killed and preserved vaccines were the least stable.”

It is generally held that the cytolysis progressively taking place in killed cholera vaccines does not lead to an accompanying loss of their antigenic properties. Several workers maintained on the contrary that the “opening-up” of the bacterial bodies enhanced the efficacy of the vaccines. Be this as it may, it is certain that the rapidly commencing process of cytolysis soon leads to changes in the aspect and density of the vaccines, thus rendering them unfit for standardization by opacity tests. Raju (1930), who made a special study of this subject, found that in the course of four weeks the average opacity standards of 29 samples of phenol-killed cholera vaccines kept at room temperature (90°F) (32°C), and of 18 such samples kept in cold storage became lowered as follows:

<table>
<thead>
<tr>
<th>Time of testing (days after preparation)</th>
<th>Average opacity in millions per ml of samples kept at room temperature (90°F, 32°C) in cold storage (32°F, 0°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (freshly made)</td>
<td>49 000</td>
</tr>
<tr>
<td>1</td>
<td>29 000</td>
</tr>
<tr>
<td>2</td>
<td>25 000</td>
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<td>3</td>
<td>19 000</td>
</tr>
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<td>4</td>
<td>18 000</td>
</tr>
<tr>
<td>6</td>
<td>16 000</td>
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<tr>
<td>14</td>
<td>15 000</td>
</tr>
<tr>
<td>21</td>
<td>13 000</td>
</tr>
<tr>
<td>28</td>
<td>12 000</td>
</tr>
</tbody>
</table>
These observations fully justify the plea of Raju that opacity tests ought to be made on the day of preparation of the vaccines and not a few days afterwards, as it was the practice in some laboratories. Raju added that, as shown by comparative tests, exposure to light exerted no influence on the alterations in opacity, but it altered the colour of the samples, giving them a brown tinge.

Though the ample observations made in regard to the keeping qualities of cholera vaccines at the time of the First World War in Europe are not always fully comparable in view of differences in the methods of preparation and of testing, it can be gathered that, in the opinion of a majority of the workers there, storage for periods of one year or even longer did not cause a loss in potency of the vaccines. Nevertheless, Hetsch (1928), commenting upon these observations, considered it inadvisable to utilize products older than six months to one year. He maintained, on the other hand, that cholera vaccines which had been stored for 3-5 weeks produced less marked reactions in man than those used immediately after manufacture.

In order to establish whether the formerly adopted policy in India to discountenance the use of cholera vaccines stored for periods longer than six months was justified, Maitra & Ahuja (1932) injected rabbits with a vaccine which had been stored for the period of one year either at 37°C or in a refrigerator at 4°C and determined the agglutinin titres in the sera of these animals. An analysis of the results showed that "a temperature of 37°C does not cause any appreciable deterioration in the agglutinogenic power of the vaccine. Agglutinin titre of freshly prepared vaccine—administered within a week of its preparation—is about the same as that of one year vaccine stored under above experimental conditions."

Though storage in the refrigerator was found to be more efficient than that in the incubator, it appeared unnecessary, therefore, to take special precautions for the preservation of cholera vaccines for periods of at least one year under temperatures not exceeding 37°C. Under these circumstances, the possibility of an extension of the storage period for such vaccines up to 12 months after their manufacture seemed to deserve serious consideration.

Evidence not only confirming but even amplifying these experiences was obtained by Taylor, Ahuja & Singh (1936) through a series of protection tests in guinea-pigs with a phenol-killed cholera vaccine, made either soon after manufacture or after storage for various periods. It was found that "vaccines stored up to two years in the plains of India and exposed to hot weather temperature of 111°F.[44°C] or over give protection practically equal to freshly made vaccine."

Hence, as far as the knowledge gained through these experiments could be applied to human vaccination, a considerable extension of the six-months' storage period for cholera vaccines appeared to be permissible even if no cold-storage facilities were available.
It is of interest to add that, in order to prolong the storage periods, a few workers such as Chiba (1922), Gutfeld (1922), and Pasricha and co-authors (1941) recommended the use of cholera vaccines which had been exsiccated. However, in view of the remarkably good keeping qualities of the usual vaccines, there seems to be no urgent call for the manufacture of such dry cholera vaccines.

**Whole fluid culture (direct) vaccines**

Though Brieger & Wassermann demonstrated in 1892 already that administration of broth cultures, which had been killed by heating for 15 minutes at 65°C, rendered guinea-pigs immune to infection with virulent cholera vibrios, it was but recently that direct cholera vaccines, prepared from whole fluid cultures, were recommended or actually used for human vaccination.

Jennings & Linton (1944) obtained by (a) cultivating *V. cholerae* under continuous aeration with a mixture of air and 20% v/v of CO₂ in a glucose-containing casein-digest medium, and (b) sterilizing the growth after an incubation for 24 hours through addition of phenylmercuric nitrate or acetate at the rate of 1 g per litre-batch of brew, a vaccine with a turbidity of 5 to 10 thousand parts per million of silica and a nitrogen content of about 0.05 g per ml. They stated that this product, injected subcutaneously in 0.1 and 0.2 ml doses into human volunteers, caused no objectionable reactions, but that the serum of these individuals was as efficacious in mouse-protection tests as the sera of persons inoculated with a cholera vaccine of the usual type. It has to be noted, however, that this claim was not confirmed by Sokhey & Habbu (1950a), according to whose comparative tests the vaccine of Jennings & Linton possessed only 1/20th of the protective power of the casein-hydrolysate vaccine described below.

As set forth by Sokhey & Habbu (1950a), the shortage of agar supplies in India during the Second World War necessitated a search for a liquid medium suitable for the manufacture of a direct cholera vaccine.

A casein hydrolysate medium, prepared according to detailed specifications of Sokhey, Habbu & Bharucha (1950) was chosen for this purpose, in which highly virulent cholera vibrios of the Ogawa and Inaba subtypes respectively were cultivated at 37°C for 3 days. 4 ml of 10% formal were then added per flask of 500 ml to make a strength of 0.08% and the flasks were again incubated at 37°C for three days. Then, after samples for testing sterility had been withdrawn, 15 ml of 0.05% phenylmercuric nitrate were added per flask as a preservative. The two monovalent vaccines thus prepared were then mixed in equal quantities and put into ampoules.

Sokhey & Habbu considered it indispensable to prepare in this manner a divalent vaccine, because in their experience (see also Sokhey & Habbu, 1950b) there was little cross-protection between the Inaba and Ogawa subtypes.
The casein-hydrolysate vaccine was standardized by a method of biological assay (Sokhey & Habbu, 1950b), to which further reference will be made below. The two workers noted in this connexion that, as shown by haemacytometer counts, their vaccine contained not more than 3000 million organisms per ml. Though admitting that this count perhaps did not represent the total bacterial content, because vibriolysis was likely to have taken place during the prolonged period of manufacture, Sokhey & Habbu drew attention to the possibility that metabolites of *V. cholerae* might play a role in immunizing mice.

Making comparative tests with their new vaccine on the one hand, with Jennings and Linton's direct cholera vaccine and three samples of agar-grown cholera vaccines manufactured in India on the other hand, Sokhey & Habbu noted that the casein-hydrolysate vaccine had a considerably higher mouse-protective power than any of the other products examined. They ascribed the low protective power of the agar-grown vaccines to a lessened virulence of the strains used for their manufacture, but claimed that "even when virulent strains were used for the preparation of agar-grown vaccines, these were found to have 1/6th to 1/9th of the protective power of casein hydrolysate vaccines made from the same strains".

Though the casein-hydrolysate vaccine has been amply used for the purpose of cholera control in India since 1945 with apparently good success, so far no large-scale statistics have become available to assess its value in the field as compared to that of agar-grown vaccines. Comparative tests on mice, made in the King Institute, Madras, with (a) the agar-grown vaccine produced in that institute, and (b) casein-hydrolysate vaccine, failed, according to Pandit (1948), to show a significant difference in the antigenicity of the two products, the animals immunized with either "withstanding approximately 100 times more of the challenge culture than the unprotected mice".

Wahba (1952) compared the efficacy of (1) an agar-grown and formol-killed vaccine containing 8000 million of cholera vibrios per ml, and (2) a fluid vaccine prepared by growing *V. cholerae* in a casamino-acid medium, which, being also formol-killed, had a titre of 5000-6000 million organisms per ml. Though mouse-protection tests showed no superiority of the fluid vaccine, Wahba stressed the simplicity of its manufacture.

Comparative tests similar in scope to those described above were made by Ranta & McCreery (1953) with (1) the agar-grown and phenol-killed cholera vaccine prepared according to Ranta & Dolman (1943) with a titre of 8000 million vibrios, and (2) a vaccine produced by cultivating the organisms in the chemically defined fluid tyrosine-asparagine-glycine medium of Ranta & McLeod (1950), diluting the growth to contain 8000 million vibrios per ml and adding phenol to a final concentration of 0.5% for the purpose of sterilization. The direct vaccine (2) protected mice
as well as the agar-grown product but was found to be less stable because it underwent rapid autolysis. Ranta & McCreery suggested, however, that it might be possible to overcome this drawback by the judicious use of formol.

It is of interest to add that Felsenfeld, Young & Ishihara (1950) recommended the sterilization of broth-grown cholera vaccines through the action of antibiotics. Neomycin was found to be most suitable for this purpose. It deserves attention in this connexion that the avirulent streptomycin-resistant cholera vibrios studied by Olitzki & Olitzki (see p. 215 above) were found to be fully antigenic and, if repeatedly injected, capable of protecting mice and guinea-pigs against several lethal doses of virulent V. cholerae.

**Vaccination with supernatants**

Making comparative tests in animals, Fairbrother (1928) found that the supernatant fluid of 24-hour-old broth cultures of V. cholerae obtained through centrifugation possessed but feeble immunizing properties, which were probably due to an incomplete removal of the organisms.

This experience was confirmed through further observations in India, Russell (1935) stating in this connexion that a

"study of the immunizing value of cholera vaccines prepared (a) from bacterial deposit, (b) from the supernatant fluid, and (c) from a mixture of deposit and supernatant fluid has revealed that vaccines prepared from supernatant fluid are not only very toxic but possess little protective value. Those prepared from the bacterial deposit are highly protective though slightly less so than vaccines prepared from the whole emulsion."

**Vaccination with culture filtrates**

As summarized by Hetsch (1912), some early workers, first apparently Vincenzi (1892), demonstrated through animal experiments that sterile filtrates of older cholera cultures in broth possessed immunizing properties.

A further noteworthy attempt to produce a cholera vaccine with the aid of candle filtration was made by Strong (1903, 1904) who kept for this purpose heat-killed suspensions of V. cholerae in saline for 3-5 days at 37°C before filtering them. It was not possible to gather sufficient experience as to the efficacy of this vaccine in the field. As shown by tests in laboratory animals and in man, the product gave rise to agglutinins and bactericidins, but exerted only a slight antitoxic action.

Analogous laboratory experiences were gained by Bertarelli (1905) with a vaccine prepared according to a method similar to that of Strong.

Besredka & Golovanoff (1923) produced a cholera "antivirus" by (a) candle-filtering cholera cultures which had been incubated for 8-10 days; (b) reseeding the filtrates with the homologous organisms and again incubating for about 8 days at 37°C; and (c) filtering once more.

When guinea-pigs which had received 1-2 ml of the final filtrate intracutaneously, subcutaneously, intraperitoneally, or intravenously, were challenged 24 hours later with 1/10th of a 24-hour-old virulent agar-slant culture of V. cholerae, the majority of the
animals survived. Controls which received in place of the cholera antiviruses plain broth or staphylococcus filtrates, succumbed rapidly.

The antiviruses was found to be heat-stable; in fact heating for 20 minutes at 100°C seemed to enhance its activity. The protection conferred by it, which in the case of intravenous administration was sometimes manifest as early as after 12 hours or even after 6 hours, remained complete for 15 days, but then disappeared in the course of the following week.

In a subsequent paper, Golovanoff (1924a) reported on comparative tests with the sera of rabbits which had been injected with antiviruses preparations and with heat-killed cholera vibrios respectively. It was found that, though endowed with immunizing properties, the antiviruses preparations did not produce agglutinins at titres higher than 1:100.

In order to show that the action exerted by the cholera antiviruses was of a specific character, Golovanoff (1924b) injected groups of guinea-pigs with filtrates prepared from \textit{V. cholerae} and heterogenous organisms respectively, and afterwards challenged all of these animals with lethal doses of cholera vibrios. The filtrates of some of the heterogenous cultures (\textit{Ps. pyocyanea}, \textit{Proteus vulgaris}, and \textit{Chromobacterium prodigiosum}) were found to confer an unspecific protection to animals challenged with \textit{V. cholerae} after 24 hours, but to none of those challenged 4 days after administration of the heterogenous filtrates. All guinea-pigs injected with cholera antiviruses survived, regardless of whether they had been challenged after one or four days.

Important immunological studies on cholera filtrates were undertaken by Singer, Wei & Hoa (1948a), who either used Seitz-filtered cultures of \textit{V. cholerae} for this purpose or obtained material from agar cultures by cutting the media, extracting the juice by pressure through several layers of cloth, and then centrifuging at high speed before resorting to filtration.

The technique used by these workers to study the activity of the filtrates was as follows:

"Guinea-pigs of approximately 300 g weight are bled, the abdomen is opened and the ileo-coecal junction is located. The ileum is excised, freed of mesentery and put into a dish with Tyrode solution. The contents of the ileum are then removed by fitting a syringe into one end of the intestine and rinsing with Tyrode solution. A glass rod of suitable size is passed through the ileum and one end of the intestine is tied to the rod. With a gentle stripping motion the intestine is inverted over the glass rod so that the epithelium faces outwards. The ileum is washed in three changes of Tyrode solution, placed on several layers of thick filter paper which have been soaked with Tyrode solution and cut into pieces of approximately 3 mm in length.

"One half ml portions of the solutions to be tested are placed in test tubes and one piece of ileum is added to each. Tyrode solution is used as diluting fluid. After incubation for one hour in the waterbath the result is read.

"When the reaction is positive the liquid surrounding the intestine becomes turbid and flocules are suspended in it consisting of epithelial cells and mucus which detach themselves from the piece of ileum when the tube is shaken. When the reaction is negative the liquid remains perfectly clear."
Exhaustive studies showed that the "filtrate factor" responsible for the above-described reactions was most regularly produced by cultivation of *V. cholerae* in beef-extract broth containing 1% agar, only irregularly in digest and peptone water media, not at all in synthetic media. It was absent from the saline washings of 24-hour-old agar cultures and the autolysates of cholera vibrios.

The filtrate factor was found to be rather heat-labile, being almost completely destroyed by exposure to a temperature of 50°C for 30 minutes. An untoward influence was also exerted by an acid reaction, storage in the incubator for one week, or addition of 0.3% formal, but not by addition of 0.5% phenol.

Discussing the significance of these findings, Singer, Wei & Hoa pointed out that the filtrate factor bore in its physical characteristics a close resemblance to a bacterial toxin. That according to Burnet and his co-workers (see the preceding chapter), the filtrate factor—called mucinase by them—was a mucin-splitting enzyme, was in the opinion of Singer and colleagues not "incompatible with its nature as a toxin as it has become increasingly probable that bacterial toxins have some of the properties of true enzymes". Nevertheless the fact that, as shown by tests with sera raised in rabbits with the aid of H + O and O cholera antigens on the one hand, and with filtrates on the other hand, a close antigenic relationship existed between the filtrate factor and the O antigen of *V. cholerae*, spoke in the opinion of Singer and colleagues against the former being identical with the cholera toxin. For, as they put it,

"All true bacterial toxins which have been described so far are antigenically specific and entirely different from the somatic antigens of the bacteria by which they are produced."

The conclusion reached by these workers was, therefore, that antigenically the filtrate factor was very similar to, if not identical with, the somatic antigen of *V. cholerae*.

Considering the practical importance of their findings, Singer, Wei & Hoa stated that:

"The discovery of F.F. (i.e. the filtrate factor) will not alter the accepted methods of cholera vaccination at present, as the antibodies produced by the somatic antigen in cholera vaccines protect the guinea-pig ileum against the effect of cholera mucinase. Preliminary experiments have shown that the sera of human subjects who have been vaccinated with cholera vaccine exert a neutralizing effect similar to the effect of immune rabbit sera."

As stated by Singer and co-authors in a second publication (1948b), they had been able to confirm through further experiments that the sera of vaccinated human subjects as well as cholera-immune sera raised in rabbits were able to protect the guinea-pig ileum against the effect of cholera filtrates. In both instances the protecting antibodies could be removed by absorbing
the sera with boiled suspensions of *V. cholerae*. The antibodies still demonstrable after absorption, being presumably of an anti-flagellar character, were unable to neutralize the factor responsible for the action of the filtrates.

It deserves attention that Singh & Ahuja (1953), to whose observations on the epithelium-desquamating enzyme of vibrios reference has already been made in the third chapter, found that strips of intestines freshly dissected from cholera-vaccinated guinea-pigs were not protected against the desquamating effect of homologous or heterologous vibrio filtrates. These two workers argued, therefore, against the claim of Singer and colleagues that cholera vaccination should confer appreciable protection against the effect of *V. cholerae* on the epithelium of the small intestine.

An important study of comparative characteristics of variously prepared cholera vaccines with regard to the preservation of mucinase in an antigenic form was recently made by Jensen (1953). For this purpose different vaccines as well as mucinase-containing solutions were used for the immunization of rabbits in order to determine the amounts of antimucinase which might be developed. It was found that immunization with cholera culture filtrates containing mucinase in an active form, gave rise not only to antimucinase at a relatively high titre, but also to agglutinins. Administration of filtrates which had been inactivated by heating for 30 minutes at 56°C, while giving rise to the latter antibodies, did not stimulate antimucinase production. Antimucinase was produced only to a low degree in rabbits which had been immunized with washed viable cholera vibrios or with heat-, phenol- or formol-killed vaccines.

Since these findings indicated that antimucinase production depended upon the presence of active mucinase in the materials used for immunization, a study of the stability of mucinase under varying conditions was made. Jensen established in this respect that:

"In a series with varied temperature-time combinations a loss of at least 75% of the activity was obtained at each of the following points: 45°C, 2 hours; 37°C, 4 hours; 21°C, 48 hours, 4°C, 2 weeks; -20°C, 50% loss in 8 weeks."

"Similar losses within 30 minutes were observed upon the addition of formalin to 0.3% and of phenol to 0.5%. With merthiolate added to a final concentration of 0.01% the losses were no greater than those recorded for preparations without preservative."

"Lyophilization gave preparations which appeared to be quite stable during an 8-week period of testing. The lyophilized material in sealed ampoules withstood the stress of 100°C for 1 hour without loss of activity upon rehydration."

Thus Merthiolate (a proprietary antiseptic known under the international non-proprietary name of thiomersal) seemed a suitable bacteriostatic agent in vaccine manufacture and lyophilization appeared to be useful for the stabilization of the products. Further, though under experimental conditions immunization with the filtrate factor gave rise to satisfactory agglutinin titres, it was in Jensen’s opinion desirable nevertheless to use for human
vaccination a killed vaccine to which mucinase had been added. To obtain such a vaccine,

"a suspension of washed V. cholerae containing $8 \times 10^8$ viable bacteria per ml was mixed with an equal volume of filtrate factor. Merthiolate was added to the mixture to a final concentration of 0.01%. This final mixture was then lyophilized, the ampoules being finally filled with dry nitrogen and glass-sealed."

Jensen stated that the mucinase titre of this vaccine was 1:1600 and that rehydrated lyophilized material gave the same titre. Since there was no detectable loss when the latter material was heated for 1 hour at 100°C, it could be anticipated that this new vaccine would keep well when stored for long periods under lower temperatures. While giving good antibody production in rabbits, it appeared to be non-toxic for these animals and for mice even upon intracerebral administration.

Further studies on the possibility of utilizing mucinase preparations for immunization against cholera were made by Freter (1955), who compared for this purpose the efficacy of (a) a killed vaccine, obtained by steaming saline suspensions of agar-grown organisms for two hours without pressure; and (b) a mucinase preparation produced by mixing the thionine-glycerol agar used for growing cholera vibrios of the same strain for 16 hours with distilled water, emulsifying the mixture and then obtaining with the aid of centrifugation a clear supernatant which was kept for use in lyophilized form. Increasing amounts (0.5-2 ml) of this preparation, standardized to a mucinase titre of 1:128, were used for intraperitoneal injection of guinea-pigs which, like those several times inoculated by the same route with the killed vaccine, were afterwards challenged through enteric infection with V. cholerae.

As Freter recorded, immunization both with the killed vaccine and with the mucinase preparation gave demonstrable protection against cholera infection. However, he added, in the case of the latter method

"it cannot be decided whether this protection is due to contamination of the mucinase used for immunization with other vibrio antigens or with other enzymes. The low agglutinin titer in protected animals which had been immunized with mucinase as compared to the agglutinin titer of protected animals which had been immunized with boiled vibrios suggests, however, that some factor other than agglutinating O-antigen in the mucinase preparations might give effective protection."

It is noteworthy that, as indicated by bacterial counts and mucinase determinations in the intestinal fluids of the animals which had succumbed to the infection, neither type of immunization was capable of altering the course of the disease once it had been acquired. Likewise the time of death after infection was not different for normal and immunized animals.

As already alluded to in the preceding chapter, Lam and co-workers (1955) adduced evidence that cholera mucinase produced a greatly increased permeability of the mouse intestine as manifested by a heightened manometric pressure within the bowel and by an increase in weight of the excised
small intestines, indicating a fluid intake into the wall of the gut. Since it was found that passive immunization of the animals against mucinase inhibited the action of the enzyme on the intestine, the comparative value of various methods of active immunization was studied, separate groups of animals being given (a) killed cholera vaccine only; (b) mucinase alone; or, finally, (c) a combination of mucinase and vaccine. It could be established in this manner that the excised intestines of mice which had been

“actively immunized with *V. comma* mucinase and tested with active mucinase gave results somewhat comparable to those obtained with passive immunization, viz., a slight increase in intralumen pressure but to a degree in no way comparable to that obtained with a preparation from an unimmunized animal. A similar result was obtained following immunization with both mucinase and chemically killed *V. comma*.

On the other hand, preparations from mice immunized with bacterial cells alone, mucinase being absent, showed a heightened sensitivity to mucinase far above that of the non-immunized controls.”

Identical results were also obtained in *in vivo* experiments in which mucinase was introduced into the small intestines left *in situ*.

An important new method for producing a fluid cholera mucinase preparation which remained stable when combined in various proportions with cholera vaccine, has recently been described by Lowenthal (1956). The salient features of processing the mucinase preparation were as follows:

Double-strength brain-heart infusion broth was dialysed at 4°C against an equal volume of distilled water for 24 hours; this process was repeated three times altogether with fresh distilled water each time. The three resulting dialysate solutions in combination were seeded with 6-hour *V. cholerae* cultures grown in the same medium and incubated at 37°C for 17 hours on a roller apparatus in a manner providing maximum aeration without excessive foaming.

After cultivation the bacterial cells were removed by centrifugation followed by Berkefeld filtration, and the filtrate was half-saturated with solid ammonium sulfate. After overnight storage in the cold room, the resulting sediment was dissolved in borate-buffered saline until the dialysate no longer precipitated with a saturated barium chloride solution. The product was then sterilized by filtration through a Selas filter.

Lowenthal established through appropriate tests that (a) the mucinase solution prepared according to his method retained its *in vitro* mucinolytic activity for periods exceeding two years; and (b) its combination with the cholera vaccine did not affect the *in vitro* activity of the enzyme, the antigenicity of the mucinase or that of the *V. cholerae* O antigen. Though admitting that “the role of cholera mucinase has not as yet been definitely established by direct evidence,” he maintained that it was advantageous for various reasons to use his purified product in combination with cholera vaccine as an immunizing agent against *V. cholerae* infection.

*Vaccines prepared from autolysates*

As will be gathered from the foregoing section, some workers, particularly Strong, though ultimately resorting to filtration when manufacturing
cholera vaccine, depended in the main upon procedures promoting what is usually called an autolysis of the vibrios. Attention has now to be drawn to some further attempts to utilize autolysates of \textit{V. cholerae} as vaccines.

Gohar (1934) resorted for this purpose to suspensions of cholera vibrios which had been kept in the laboratory for several weeks until all organisms had died and most of them had become lysed. Used directly for the immunization of guinea-pigs, this autolysed vaccine appeared to be more effective than the vaccines killed by exposure to a temperature of 60°C.

Violle (1950) found that the lysate of cholera vibrios which he was able to produce with the aid of supersonic vibration (see Chapter 3, p. 161) possessed moderate antigenic properties. In his opinion this method would not be suitable for the purpose of practical vaccine manufacture. However, the manufacture of a cholera vaccine through application of supersonic vibration has been recorded by Bosco (1955).

\textit{Vaccines prepared by extraction methods}

Attempts to use extracts prepared in various ways from the organisms for the purposes of cholera vaccination have been made by numerous workers. While, as described below, most of them resorted to chemical procedures, a few implemented mechanical methods, such as expressing the “plasmatic juice” of previously ground-up vibrios (Hahn, 1897) or breaking up the bodies of the organisms at the temperature of liquid air (Macfadyen, 1906). However, Hahn’s vaccine, though found to confer a long-lasting immunity to experimental animals, was apparently never used for human vaccination, while the antigen prepared by Macfadyen served only for serum manufacture.

To prepare antigens suitable for cholera immunization by chemical methods, some workers, first apparently Klebs (1892), resorted to alcohol extraction. Gohar (1934) used distilled water to extract cholera vibrios which had been dried \textit{in vacuo}. To judge from guinea-pig experiments, addition of such extracts to heat-killed cholera vaccines enhanced the protective power of the latter.

Gohar & Isa (1948) stated that they had prepared a soluble extract from cholera vibrios

\begin{quote}
"by adding to a suspension containing 8,000 million organisms per ml an equal quantity of normal sodium hydroxide and incubating at 37°C, for a few hours until the organisms are dissolved and the suspension becomes clear. This is subsequently neutralized with HCl until it is just alkaline (pH about 7.5)."
\end{quote}

Fifteen out of 25 rats, which had been twice injected with this extract at a week’s interval, survived intraperitoneal challenge with LD$_{50}$ doses of living cholera vibrios.

The possibility of using the nucleoproteids of \textit{V. cholerae} referred to above for the purposes of vaccination was experimentally explored by several workers, first by Heller (1905), Schmitz (1906), and Biell (1906).
Though in the opinion of Heller and of Blell nucleoproteids were suitable for human cholera vaccination, Hetsch (1912) stressed on the contrary that, in view of their high toxicity, the mediocre titre of the antibodies produced by them; and the technical difficulties of properly manufacturing them, administration of the usual vaccines was far preferable. It appears in fact that vaccination with nucleoproteids has never been used for the purpose of protecting man against cholera infection.

**Toxoids**

The use of toxoids prepared from 10-day-old El Tor cultures by addition of 0.5% formol and storage for eight days was recommended for human vaccination against cholera by Kraus & Kovacs (1928), because in their experience (a) subcutaneous injection of these products into rabbits and guinea-pigs protected these animals not only against one or several lethal doses of the El Tor exotoxin but also against intraperitoneal administration of living El Tor or cholera vibrios, leading as well to agglutinin production in the sera of these animals, and (b) the reactions produced by these toxoids in man were not marked.

Felsenfeld & Young (1945) experimenting on rabbits, guinea-pigs, and mice with differently prepared cholera vaccines, obtained the best results by using formol- or phenol-killed cholera vibrios of the Inaba subtype combined with formalized filtrates of Inaba or El Tor vibrios. They preferred the latter organisms for preparing such toxoids because these were less toxic and stimulated the production of antibodies against the haemolytic and necrotic action of *V. cholerae* to a higher level. The sera of human volunteers tested with this toxoid-vaccine, which was used in combination with a dysentery vaccine, were found capable of protecting mice against cholera infection.

In order to obtain a cholera toxoid, Gohar & Isa (1948) treated the soluble extract they had obtained from suspensions of *V. cholerae* through the addition of sodium hydroxide (see page 317) with 0.7% formol and incubated the mixture for 20 days at 37°C. While the lethal dose of the original soluble extract for rats was 3.5 ml subcutaneously, the formol-treated product was almost atoxic and rendered animals immunized with it resistant to large toxin doses. Used experimentally for the vaccination of rats which were afterwards challenged with LD₅₀ doses of *V. cholerae*, mixtures of vaccine and toxoid gave better results (84% survival) than toxoid alone (56% survival), the toxic extract or killed vaccines.

Following up preliminary trials by Gohar & Isa, Gohar (1948) experimented with an alum-precipitated cholera endotoxoid prepared by

(a) cultivation of *V. cholerae* for 2-3 days in broth filled into flat flasks, which were placed horizontally to provide a maximum of aeration; (b) addition of 0.5% formol followed by further incubation at 37°C for a week; (c) precipitation with 0.5% alum;
and (d) collection of the precipitate and resuspension to the required density in phenolized normal saline.

Results obtained by single administration of this vaccine in mice, which were afterwards challenged with LD\textsubscript{100} doses of living cholera vibrios, were almost as good as those produced by two administrations of a heat-killed vaccine (40% as against 45% survival).

Tested on a small scale in man, the alum-precipitated vaccine produced, when injected intradermally, indurated masses which persisted for a long time and were, in Gohar's opinion, thus capable of exerting an antigenic stimulus lasting for several days. Administration of full doses subcutaneously produced somewhat severe reactions. These observations speak against the practicability of this method of cholera vaccination, recommendable though it might be on theoretical grounds.

\textit{Sensitized vaccine}

The use of sensitized cholera vaccines has been recommended by Japanese workers, first apparently in 1916 by Takano and by Yabe (see Shiga, Takano \& Yabe, 1918; Takano, Ohtsubo \& Inouye, 1926). The method used by Takano for the preparation of such vaccines has been summarized by Takano and colleagues as follows:

"A 20-hours agar culture 1 gm. is suspended in 2 c.c. of a cholera immune horse serum (bactericidal titre of at least 0.0001 c.c.) diluted four times with the salt solution; the mixture is then incubated for 2 hours during which it is shaken from time to time. Then the suspension is centrifuged at very high speed and the organism is washed twice with salt solution. The organism thus sensitized is suspended in physiological salt solution containing 0.5% of carbolic acid. Of such a suspension 1 c.c. contains 2 mgm. of the organism. The cholera vaccine thus prepared is placed in an incubator overnight... An essential part of this process is to prevent the death of the cholera vibrio until the sensitisation is complete. If dead vibrios were to be used, the antigen would be largely set free in the medium and lost in the process of washing, so that the antigenic power of the vaccine would be greatly reduced."

In contrast to these recommendations, a few workers, particularly Besredo (1922) and Masaki (1922a) advocated the use of living sensitized cholera vaccines, but these—though found satisfactory in the laboratory—seem not to have been utilized for human vaccination. Sensitized vaccines prepared according to Takano's method have been used on a fairly large scale in Japan with satisfying results, it being claimed in particular that in contrast to the usual type of cholera vaccines the sensitized products caused but mild reactions, yet produced an immunity which set in rapidly and which reached a considerable degree even after single doses only had been administered. Since, however, the difficulties of preparing sensitized vaccines on a scale sufficient for mass campaigns are enormous it is not surprising to find that in spite of their undeniable advantages the use of such products has been given up entirely.
Cutaneous vaccination

Making parallel observations on guinea-pigs which had been injected intraperitoneally at eight days’ interval with two doses of a heat-killed cholera vaccine and on a second group of animals which had the same amounts of vaccine (0.3 and 0.6 ml) rubbed into their freshly-shaven skin, Ciucu & Balteanu (1924a) found that the animals of both groups resisted intraperitoneal challenge with a lethal dose of V. cholerae made 12 days after the second vaccine administration. However, it was found that only the intraperitoneally vaccinated guinea-pigs and not those protected by the percutaneous route showed agglutinins, bacteriolysins, and complement-fixing antibodies in their sera. Further testing the response to intracutaneous injection of a suspension of live cholera vibrios in 0.2-ml amounts, Ciucu & Balteanu (1924b) noted (a) a marked skin reaction in normal guinea-pigs; (b) a less marked reaction in animals protected by two intraperitoneally administered doses of cholera vaccine and tested 12 days after the second injection; and (c) practically no skin reaction in the animals which had been vaccinated by the percutaneous route. Ciucu & Balteanu claimed on account of these findings that percutaneous cholera vaccination produced a local cellular immunity.

Interesting as this postulation is, it has to be noted that according to all workers who subsequently devoted attention to this point (see Neuhaus & Prausnitz, 1924, for example), intracutaneous administration of cholera vaccines did lead to the appearance of antibodies in the sera of the vaccinated animals. Panja & Das (1947) as well as Singer, Wei & Hoa (1948b), who made corresponding observations in man, found that antibody formation was apt to take place in the intradermally vaccinated persons to a more marked degree than was the case in subcutaneously vaccinated individuals.

Commenting upon their experiences when vaccinating 11 persons intradermally with 0.1 ml and 0.2 ml of the standard Kasauli vaccine and 10 individuals (controls) subcutaneously with 0.5 ml and 1.0 ml doses of the same vaccine, Panja & Das lauded the great economy in material effected by the former method and also stated that the reactions produced by intracutaneous vaccination were negligible. They admitted, however, that in mass campaigns it was considerably less expedient to use the last-mentioned in place of the subcutaneous method.

Singer, Wei & Hoa (1948b) noted the appearance of small abscesses at the site of the intracutaneous injection in some of the persons vaccinated three times with 0.2-ml doses. Since, however, such abscesses became manifest only at the third injection, they were probably the result of an allergic reaction and might be avoided by administering only two doses at 5-7 days’ interval. Be this as it might, no doubt one must share the misgivings of Panja & Das regarding the extrinsic difficulties of using the cutaneous method of cholera vaccination in mass campaigns.
Vaccination by the nasal route

Sanarelli (1924b) found that insufflation of a powder consisting of toluene-killed cholera vibrios and boric and lactic acid into the nasal cavity of rabbits led to an often quite considerable formation of agglutinins in the sera of the animals and rendered them immune to cholera infection by the intravenous route.

Oral vaccination

While Brüger, Kitasato & Wassermann (1892) reported that they had rendered guinea-pigs immune to oral infection with *V. cholerae* by intraperitoneal administration of heat-killed vaccines, Klemperer (1892) claimed that it was also possible to protect the animals against oral infection by the introduction of small doses of living cholera cultures into their stomach. Similarly, Cantacuzène (1894) reported that repeated introduction of cholera vibrios into the stomach of guinea-pigs rendered the animals fully resistant to intraperitoneal infection with *V. cholerae*, provided that they were challenged not earlier than 18 days after the last intragastric inoculation. However, Sobernheim (1893), though finding that intragastric immunization with rather large doses of cholera vibrios conferred some degree of protection against subsequent intraperitoneal infection, was unable to protect guinea-pigs against oral infection either by the intragastric or any other method of cholera vaccination.

Pfeiffer & Wassermann (1893), who infected a considerable number of immunized guinea-pigs *per os* with recently isolated cholera vibrios found similarly that

"the percentage of immunized guinea-pigs which survive this mode of infection is not appreciably higher than in the control guinea-pigs. The mode of immunization seems to exert no influence in this respect. We found no difference when we immunized the guinea-pigs with living or killed cultures, subcutaneously or intraperitoneally, when we challenged a few days after immunization or waited for weeks." [Trans.]

Sawtschenko & Sabolotny (Zabolotny) reported in 1893 upon observations they had made when orally administering numerous doses of an agar-grown and heat-killed cholera vaccine during about a month to themselves and during about two weeks to a student. The total amount of vaccine taken by Sawtschenko was 180 ml, equalling 1.4 g of cholera vibrios weighed in the dry state, while Zabolotny ingested 110 ml (dry weight of vibrios about 0.84 g) and the student 135 ml (dry weight of vibrios about 1 g). The sera of Sawtschenko and the student, when administered intraperitoneally to guinea-pigs in doses of 0.1-1 ml, 25 days after immunization had been completed, protected the animals against challenge with two lethal doses of *V. cholerae* made three days afterwards.

After they had partaken of further vaccine doses, bringing the total dry weight of cholera vibrios administered orally to about 1.7 g and 2.3 g
respectively, Sawtchenko & Zabolotny ingested after previous alkaliniza-
tion of their stomach content 0.1 ml of a 24-hour-old virulent cholera
broth culture. Though it was possible to demonstrate the presence of
the organisms in the stools of Zabolotny up to three days after infection
and in the faeces of Sawtchenko on the second day, neither of them showed
any clinical signs of the disease.

Remarkable though these experiences are, it has to be pointed out that
(a) even the attempts to produce cholera artificially through oral infection
of non-vaccinated persons were by no means always crowned with success,
and (b) in view of the prolonged course of oral vaccination with enormous
doses, the results of the two Russian workers did not furnish any proof of
the practicability of this mode of cholera immunization.

In a further report made in 1894 Zabolotny stated that he had been
able to protect sisels (Spermophilus guttatus) against intragastric as well
as against intraperitoneal cholera infection through administrations,
repeated several times, of live attenuated or heat-killed V. cholerae cultures per os —
results which were afterwards confirmed by Korobkova (1922). Subcutaneous
or intraperitoneal immunization of sisels with killed cholera vibrios on
the contrary failed to protect the animals against intragastric infection.

In analogy with the last-mentioned observation, Metchnikoff (1894)
stressed that he found it impossible to protect young unweaned rabbits by
parenteral administration of either living or killed cholera vibrios against
oral infection with V. cholerae, which produced in such animals a process
apparently identical with that observed in human victims of the disease.
These negative results were confirmed in 1911 by Choukevitch. As sum-
marized by Hetsch (1912), analogously disappointing results were also
obtained by some other workers with dogs and cats.

The possibility of conferring immunity to cholera through intragastric
administration of Galeotti’s (1912) nucleoprotein was explored by de Bonis
& Natale (1913). They found that only two out of 11 guinea-pigs which had
been given with the aid of a stomach tube 1-3 doses of 0.005-0.02 g of
cholera nucleoprotein dissolved in 0.5% sodium bicarbonate solution,
survived this treatment. They were able to establish, however, that (a)
the sera of 9 of these animals contained agglutinins (maximal titre 1:1000),
and (b) the two survivors resisted infection with 0.25 ml of a cholera broth
culture, while a control given the same dose died in 48 hours.

The problem of oral cholera vaccination received far more attention
than in the past after Besredka, in a series of articles published in 1918 and
1919 in the Annales de l’Institut Pasteur had reported upon successes
obtained in the experimental prevention of dysentery, typhoid and para-
typhoid by the combined administration of ox-bile and vaccinating doses
of the organisms in question by the oral route. Masaki (1922b) investigating
whether these observations were applicable in the case of cholera, thus
summarized the findings he had made in this respect:
"(a) Both rabbits and guinea-pigs are completely refractory to the ingestion of cholera vibrios in any dose.

(b) Oral administration of bile alters the intestinal wall of rabbits, facilitates the entry of the cholera endotoxin and its passage into the system: as a consequence agglutinins appear in the animals which had ingested either living or killed vibrios after bile sensitization.

(c) Ingestion of either living or killed vibrios does engender protective antibodies in sensitized as well as in non-sensitized rabbits.

(d) Only bile-sensitized rabbits react to the ingestion of living vibrios: very high doses (two agar cultures in Roux bottles) kill the animals in one to two weeks; median doses (one culture) render the animals ill for some days; doses less than half a Roux bottle finally cause no harm.

(e) Only bile-sensitized animals which have shown illness after the ingestion of living vibrios, become vaccinated against intravenous administration of a surely lethal dose of vibrios." [Trans.]

Masaki added that the immunity engendered in this manner was in all probability a local (intestinal) one. In this connexion, he laid stress upon the fact that, though ingestion of bile followed by that of living cholera vibrios led to the appearance of agglutinins in the sera of the rabbits, these antibodies, instead of augmenting, decreased and finally disappeared, apparently because administration of the initial vibrio doses had led to a "vaccination" of the intestinal wall which thus became impermeable to the organisms or their products.

The validity of Masaki's conclusions was supported by some laboratory observations, e.g. those of Glotoff (1923) and of Horowitz-Wlassowa & Pirojnikova (1926), but some other workers, such as Sdrodowsky (Sdrodowski) (1924) and Kliichin & Viggotschikoff (1925) took a definite stand against the method of oral cholera vaccination. Engelhardt & Ray (1927) concluded from an exhaustive study that it was possible to immunize bile-sensitized rabbits by oral administration of very large doses of living cholera vibrios against intravenous infection. The agglutinin titre in the sera of these animals rose but slightly and then decreased. In the rabbits which were orally given killed cholera vibrios after bile administration, no immunity resulted but the agglutinin titre of their sera rose constantly during immunization and the following two weeks.

In view of these discrepant results it is not surprising to find that the opinions held by the different observers in regard to the question of whether cholera immunization per os led to a local or a general immunity, were rather divided. Some, for instance Horowitz-Wlassowa & Pirojnikova, were in favour of the former view, but others, Sdrodowsky for example, denied the existence of a separate enteric immunity of histogenous origin—an opinion also vigorously expressed by Hetsch (1928).

On account of the observations he had made in the past with Sawtschenko, Zabolotny (1922) advocated the large-scale use of oral
vaccination for coping with the cholera situation in Russia. As summarized in the *Tropical Diseases Bulletin* (1923), he

"recommends the use of vaccines prepared from thick suspensions of organisms killed by heat, carbollic acid or alcohol (20-40 per cent.), from 3 to 5 doses of 7-10 c.c. every other day. Each dose contains from 10 to 100 milliard vibrios, or from 0.01 to 0.1 gm. of dried organisms. Vaccines were also prepared in the form of tablets with sugar or cocoa, each containing 0.1 gm. of dried organisms."

Zabolotny stated that, as shown by preliminary experiences, in persons who had been immunized against cholera in this manner, the agglutinin titre rose to 1:400 and the bactericidal titre to 1:60. His article does not indicate whether large-scale advantage of oral cholera vaccination was taken in Russia.

Among the studies made during the years following Zabolotny's publication in regard to the appearance of antibodies in the sera of individuals orally vaccinated against cholera, the following deserve mention:

Korobkova & Zenine (1923) tested the sera of 49 out of 348 persons who had been immunized by oral administration on each of 3 subsequent days of one tablet respectively containing 50 milliards (US billions) of heat-killed cholera vibrios, 115 of these individuals also receiving on each occasion a bile tablet. In the sera of 19 individuals, which were examined 17 days after immunization, an agglutinin titre of 1:100 (the maximum tested) was found to be present invariably, regardless of whether or not bile tablets had been given.

In 30 persons, whose sera were tested 5 weeks after immunization, agglutinins were but rarely demonstrable, but bacteriolysins at titres ranging from 1:10 to 1:25 were found to be present with one exception, both in the group receiving vaccine only and in that receiving vaccine and bile tablets. In Korobkova's opinion it was therefore uncertain whether bile administration had to be combined with the oral administration of cholera vaccines. The same doubt was also expressed by Peverelli (1924).

Gluchow and co-workers (1923), testing the sera of 73 individuals vaccinated orally, noted the appearance of agglutinins and bacteriolysins which persisted for 9 months, but decreased in the majority 4 months after vaccination to half the titre. A repetition of oral vaccination did not lead to an increased antibody titre, but such an increase was noted after the subcutaneous administration of booster doses. In the opinion of the above-mentioned workers these observations supported the view that oral vaccination created a barrier against the passage of vibrios or their products through the intestinal mucosa, which could be circumvented through parenteral revaccination. This view was opposed by Stepanoff-Grigorieff & Iljina (1924), in whose opinion the appearance of agglutinins in practically all persons orally vaccinated and that of bacteriolysins in part of these individuals manifested the development of a general immunity.

Far more important than the experiences recorded above were large-scale trials of the method of oral cholera vaccination in India, made simultaneously with mass vaccinations by the subcutaneous route.

The procedure adopted in these campaigns for oral vaccination was to administer on each of three consecutive mornings, before food had been taken, first a bile tablet and 15 minutes later a commercially prepared bilivaccin tablet with a bacterial content of

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1 As reported by Sarramon (1930) a comparative study of oral and parenteral cholera vaccination was also made in Indochina. It showed attack rates of 0.36% in the 4982 persons who had received bilivaccin and of 0.37% in 8485 individuals vaccinated parenterally as against attack rates of 2.02% and 1.67%, respectively in the two control groups of 11,004 and 29,254 persons.
about 70 milliards (billions) of dried cholera vibrios. Parenteral vaccination consisted of the subcutaneous injection of either one or two doses of a standard cholera vaccine with a vibrio content of 8000 million.

Reporting on the first of these trials, Russell (1928a, 1928b) submitted the following figures:

<table>
<thead>
<tr>
<th></th>
<th>Cholera attacks</th>
<th>Cholera deaths</th>
<th>Percentage recovered</th>
<th>Percentage mortality among attacked</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. <strong>Bilivaccin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number given 3 doses of bilivaccin</td>
<td>4982</td>
<td>18</td>
<td>4</td>
<td>0.36</td>
</tr>
<tr>
<td>Number not treated (controls)</td>
<td>11004</td>
<td>222</td>
<td>93</td>
<td>2.02</td>
</tr>
<tr>
<td>B. <strong>Cholera vaccine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of persons given one dose (0.5 ml)</td>
<td>17160</td>
<td>59*</td>
<td>25*</td>
<td>0.34</td>
</tr>
<tr>
<td>Number of persons given two doses (1.5 ml)</td>
<td>8485</td>
<td>31</td>
<td>2</td>
<td>0.37</td>
</tr>
<tr>
<td>Number of persons not treated (controls)</td>
<td>25645</td>
<td>489</td>
<td>184</td>
<td>1.67</td>
</tr>
<tr>
<td>* Attacks and deaths occurring within three days after vaccination excluded.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

While these figures, besides illustrating the value of the usual method of cholera vaccination even with a single 0.5-ml dose, demonstrate also the efficacy of oral vaccination, it has to be noted that in the experience of the field staff, "the bilivaccin sometimes produced acute diarrhoea of such a severe type that the persons affected refused to take further doses, and, in certain cases indeed, the medical officers were accused of inducing cholera. Fortunately, no untoward incident occurred, as those affected quickly recovered."

Submitting the gross figures quoted above and the subsidiary statistics to a painstaking analysis, Russell reached the conclusion that

"[a] the immunity developed five days after a single dose of anti-cholera vaccine is nearly as high as that conferred three days after a full course of oral bilivaccin;"

"[b] ... it may be inferred that a high degree of immunity is conferred by both the subcutaneous anti-cholera vaccine and the oral bilivaccin, but that the former is, in the long run, superior to the latter. In view of the fact that, with ordinary precautions, the risk of injury from inoculation is inappreciable and that even transitory discomfort is uncommon, the case in favour of anti-cholera vaccine as a practical and cheap preventive measure is complete."

As stated by Russell (1935), during 1932 another large field experiment with bilivaccin and with anti-cholera vaccine was carried out in endemic-cholera areas of Madras Presidency (now Madras State), several thousand persons being protected by the former method and an additional 6000 persons by the latter. The conclusions reached after statistical analysis were that

"(1) both the full three-dose course of bilivaccine and the 1 c.c. dose of anti-cholera vaccine conferred a considerable degree of protection against cholera, and
"(2) the incidence of cholera amongst the unprotected was 8.5 times higher than among those protected by bilivaccine (3 doses) and 5.5 times higher than amongst those protected by anti-cholera vaccine."

In Russell's opinion these observations seemed to confirm those made in the first field study. He added:

"The question of the substitution of bilivaccine for anti-cholera vaccine for the protection of Haj pilgrims was recently referred to the 'Office International' for an expression of opinion, but that body has declared that while vaccination per os probably produces a certain immunity, this is much inferior to that obtained by subcutaneous inoculation. Moreover, the difficulty of exercising a strict control appeared to the 'Office International' to be sufficient reason for rejecting the suggestion."

There can be no doubt that the almost insurmountable difficulties of properly using oral cholera vaccination under the conditions ordinarily prevailing during mass campaigns as well as the unpleasant and sometimes even serious reactions apt to follow bile administration strongly speak in favour of parenteral immunization. It was probably for these reasons as well as on account of the difficulty and costliness of preparing oral vaccines that, after having received much attention for some time, the method of cholera vaccination per os has been given up entirely.

**Mixed vaccines**

Castellani, who seems to have been the first worker to draw attention to the possibility of using mixtures of vaccines manufactured individually from different bacterial species for simultaneous immunization against the respective infections, suggested in 1913 that combined administrations might be made of vaccines prepared from live attenuated cholera vibrios and dysentery bacilli. He and Mendelson (1915) followed this proposal by recommending the use of a tetra-vaccine obtained by mixing vaccines prepared separately from typhoid, paratyphoid A and B bacilli, and from cholera vibrios, the finished product containing per ml 500 million of the first mentioned organisms, 250 million each of the two paratyphoid strains, and 1000 million of *V. cholerae*. It is of interest that 0.5% phenol alone was used to sterilize these vaccines, a storage of the phenolized suspensions at 10°-20°C for a few hours being found sufficient for this purpose. Dealing exhaustively with the use of various combined vaccines, Castellani (1916) recommended *inter alia* a mixed cholera and plague vaccine and a "penta-vaccine" for simultaneous immunization not only against these two infections but also against typhoid and paratyphoid A and B.

As summarized by Hetsch (1928), numerous European workers recommended and to some extent practised combined vaccinations against typhoid and cholera at the time of the First World War, while in the Philippines Manalang (1925) made ample use of single-dose administration of a tetra-vaccine prepared according to Castellani's method, which contained
per ml 4000 million cholera vibrios, 2000 million typhoid bacilli, and 1000 million each of paratyphoid bacilli A and B.

More recently, Gefen (1945) recommended a polyvalent vaccine prepared with the aid of extraction methods for simultaneous vaccination against cholera, typhoid, paratyphoid, dysentery, and tetanus. Similarly, Ranta & Dolman (1943) recorded favourable results of laboratory tests (production of agglutinins in rabbits) with a combined vaccine containing 4000 million of cholera vibrios, 700 million of typhoid bacilli, 225 million respectively of paratyphoid bacilli A and B per ml of tetanus toxoid. Felsenfeld & Young (1945), to whose method of manufacturing a toxoid-vaccine against cholera reference has been made above, tested a mixture of this and a similarly prepared dysentery vaccine on a group of volunteers and obtained satisfactory results when using the sera of these individuals for serological and mouse-protection tests.

While in some instances the combined vaccines were issued ready-made by the manufacturing laboratories, some workers mixed the individual vaccines they proposed to administer in combination immediately before use. Schwarz (1919) was not satisfied with either of these procedures, fearing on the one hand that storage of combined vaccines might lead to a deterioration of their immunizing power and, on the other hand, that an instantaneous mixture of individual vaccines might lead to contaminations. He advocated, therefore, mixing the vaccines destined for combined administration two days before their use, so that the phenol or other antiseptic contained in them could cope with contaminating organisms.

Judging from experiences gained through laboratory tests, mainly those with the sera of persons to whom the combined vaccines had been administered, the various workers using such products were unanimous in asserting their efficacy. Indeed, some observers, e.g., Manalang (1925), maintained that the combination of different vaccines exerted a stimulating influence on the immunizing power of the individual components. It was also generally held that the reactions caused by the administration of mixed vaccines were not more marked than those following the separate use of the single components of these products. However, fairly ample experience of combined cholera and typhoid-paratyphoid vaccination in China has convinced the present writer that the latter claim holds true only to a limited extent: while the reactions produced by these mixed vaccines were not more marked than those caused by the administration of typhoid-paratyphoid vaccines alone, they were much stormier than those resulting from the sole use of cholera vaccines. Hence, while the absence of marked reactions was instrumental in overcoming the prejudice against sole cholera vaccination, the use of the combined vaccines invariably led to complaints seriously hampering the campaigns. In the considered opinion of the present writer it is not advisable, therefore, to use mixed vaccines in the course of general anti-cholera campaigns, the less so because as a rule these have been
prepared with substandard amounts of cholera vibrios—a drawback which becomes particularly serious when only single vaccine doses can be administered. One must admit, however, that under special conditions, particularly when applying vaccination methods for the protection of armed forces, the advisability of using combined vaccines of an adequate standard deserves consideration.

**Negative phase**

As in the case of other infectious diseases, so also in that of cholera it has been asserted by some workers that vaccination is followed by a negative phase during which the susceptibility of the immunized to the infection is temporarily increased. As can be gathered from a study of the literature, particularly the statements of Pfeiffer & Friedberger (1908b), Aaser (1910), Bessau & Paetsch (1912), Papamarku (1917), Schwartz (1919), and Hetsch (1928), these claims were mainly based on the one hand on observations of changes in the titre of immune bodies in the sera of the vaccinated and on the other hand on experiences regarding the incidence of cholera in recently immunized individuals.

The claims made by some observers that a negative phase was created through a drop in the antibody content of the sera of the vaccinated deserve no credence, not only because such a lowering of the antibody titres has not been confirmed by other workers but also because the laboratory experiences discussed below clearly show that cholera vaccination is not followed by a phase of temporarily increased susceptibility to the infection.

Exhaustive studies made in this respect by Pfeiffer & Friedberger (1908b) showed that experimental animals which had been immunized with specific vaccines, instead of becoming increasingly susceptible to cholera during the period following immediately, showed on the contrary at once a resistance—probably at first an unspecific resistance—to the infection. Bessau & Paetsch (1912), continuing these studies, were also unable to demonstrate the presence of a negative phase through animal experiments performed “under conditions which had to be considered very favourable in comparison to human immunization”. Attention has been drawn already (see page 251) to further observations by Papamarku (1917), who showed that the drop in bactericidal power observable in the sera of guinea-pigs after cholera revaccination was as a rule not accompanied by a loss of resistance to challenge infection.

When it is considered that (a) as generally agreed, a period of at least about three days has to elapse before the immunity engendered by active cholera immunization begins to become manifest, and (b) during outbreaks it is inevitable that some persons, because they are incubating the disease at the time of vaccination, fall ill before being protected, it is easy to understand why, as soon as Haffkine’s method of vaccination began to be practised, its many adversaries clamoured that this procedure, instead of pre-
venting, caused or at least facilitated the appearance of cholera. Haffkine took a determined stand against the idea of a negative phase, stating for instance in a speech given in 1899 before the Royal Society, London, in reference to some of his early statistics that:

"Inoculation has again acted, so to say, immediately; or as we have adopted to generally formulate the result, has acted within the time necessary for the subsidence of the general reactionary symptoms produced by the inoculation."

In spite of these and other reassurances, the idea of a negative phase following cholera vaccination in man continued to be ventilated from time to time, for instance as late as in 1950 by Dani. However, a large majority of the cholera workers considered the appearance of the disease in quite recently vaccinated persons merely a post hoc and not a propter hoc phenomenon. Thus Simpson (1915), referring to careful observations made in this respect in India, denied that vaccination against cholera led to a negative phase in man and his opinion has been endorsed by many workers dealing with this infection in Central Europe during the First World War. Petrovich (1915) even stated that he had used cholera vaccine with good success for the treatment of a quite considerable number of patients who had been attacked by the disease. It was also pointed out with much reason by several workers (see Hetsch, 1928) that the non-appearance of clinical signs of the disease in specifically vaccinated carriers of V. cholerae strongly spoke against the appearance of a negative phase. More important still, a large-scale statistical study by Adisesan, Pandit & Venkatraman (1947), to which full attention will be paid in the tenth chapter, failed to show that the administration of a standard cholera vaccine in single doses led to a significantly increased incidence of the disease among the inoculated.

The evidence adduced above suffices to show that one should not hesitate in emergencies to make ample use of cholera vaccination even during epidemics. At the same time it is clear, however, that both in order to benefit as many persons as possible and to avoid alarming the people by the occurrence of the disease in recently vaccinated individuals, every possible effort should be made to administer the vaccinations before onset of the cholera seasons.

Duration of immunity

While, as discussed above, general agreement exists that active cholera immunization does not confer protection against the infection during the days immediately following administration of the vaccine and it is also usually held that a period of about a week has to elapse before a substantial immunity becomes established, opinions regarding the duration of the immunity vary. Haffkine, summarizing the experiences with his vaccines up to 1906, maintained in this connexion that

"their effect becomes rapidly accentuated during the first few days and lasts, when moderate doses are used, for about 14 months, after which time it begins to decrease markedly and probably to disappear".
Though some workers stated that cholera vaccines manufactured according to Kolle's method conferred immunity for a year, others maintained that the period of protection afforded by killed cholera vaccines lasted only for 7-9 months, or merely for 6 months or even less. Adiseshan, Pandit & Venkatraman (1947), summarizing the observations they were able to make in the course of their above-mentioned statistical study, stated in this connexion that:

"In the villages which had second outbreaks within the first six months after the first outbreak, the incidence of cholera in persons who had been inoculated at the time of the first outbreak is definitely lower than in the un inoculated. These differences are statistically significant. The protection afforded by anticholera inoculation continues, therefore, for at least six months.

"Although the figures available are too small to warrant a definite conclusion, the virtual absence of cholera among inoculated persons in villages which were re-infected between six and twelve months after the first outbreak suggests that immunity may last for as long as twelve months."

It follows from these observations that in localities were cholera becomes, or is apt to become, epidemic perennially, at least yearly revaccinations are indicated. Hetsch (1928), summarizing the experience acquired regarding the duration of the immunity after cholera vaccination during the First World War, was of the opinion that as long as a danger of infection continued to exist, 0.5-ml booster doses of cholera vaccines ought to be administered at half-yearly intervals—a procedure found satisfactory in the German and Austrian armies.

Evaluating tests

It is essential to state that the tests used to assay the immunogenic value of bacterial vaccines in general, and of cholera vaccine in particular, form only part of the examinations necessary to ascertain the suitability of these products. It is of prime importance to determine on the one hand that the finished products are of proper standard and to ascertain on the other that they are free from aerobic or anaerobic contamination and, in the case of killed vaccines, also that the method of sterilization used has been effective. Unless it is incumbent upon a laboratory to assay commercially produced vaccines, the implementation of the tests just mentioned forms a part of the routine followed for vaccine manufacture.

A further preliminary step of great importance is to ascertain that the cholera vaccines issued produce no undue reaction on account of either an exalted toxicity or of too high a content in phenol or other antiseptics. Pasricha, Chatterjee & Paul (1938) recommended that the absence of an unduly high toxicity be proved by the survival of guinea-pigs given 5-ml doses of the products under test intraperitoneally, and that the absence of an excess of antiseptics be demonstrated by showing that adult mice which had been subcutaneously injected with 0.5-ml doses of the vaccines examined remained free from serious symptoms during the period of one week.
The important methods available in practice for assessing the immunizing properties of cholera vaccines may thus be classified:

(1) direct agglutination tests with the vaccines as antigens and standard cholera-immune sera;

(2) serological tests (including Pfeiffer tests) to ascertain the presence of antibodies in the sera of actively immunized animals or vaccinated human subjects;

(3) active immunization experiments in laboratory animals;

(4) protection tests with the sera of vaccinated animals or human subjects.

The importance of these various methods will now be dealt with seriatim.

(1) Direct agglutination tests. Summarizing the experiences of Pasricha and co-workers (1938, 1941) when examining numerous cholera vaccines of different origin, Taylor (1941) stated that these workers took advantage of three categories of tests, namely (a) direct agglutination of the finished vaccines with pure O sera of the Inaba and Ogawa subtypes; (b) agglutinogenic tests in rabbits; and (c) protection tests in guinea-pigs. Taylor emphasized that:

"The results of these tests were found to run parallel to each other and when a vaccine did not agglutinate to satisfactory titre with the O sera no protection against an infecting dose of V. cholerae was obtained."

Endorsing on account of these experiences the great value of direct agglutination tests, Taylor concluded, therefore, that "if a vaccine is sterile and shows satisfactory agglutination it can be considered satisfactory".

(2) Serological tests. As has been stated before, agglutination tests with the sera of immunized animals and more still with those of vaccinated human beings have been made by numerous workers but have yielded rather discrepant results. There can be no doubt that, besides showing up differences in the character of the various vaccines examined, these discrepant results were due to a large extent to extrinsic causes, particularly (a) differences in the size and number of the vaccine doses administered, and (b) differences in the technique implemented by the various workers, especially the use of less suitable killed antigens in place of live cholera vibrios. At the same time it must be admitted, however, that even if the tests are performed in an adequate manner with the sera of suitably vaccinated individuals, agglutinins, if at all demonstrable, appear at different and not rarely rather insignificant titres and persist for different periods, not necessarily coinciding in length with those during which immunity is supposed to last.

While in the opinion of some workers these inconstancies sufficed to render agglutination tests with the sera of vaccinated subjects of little value for the assay of cholera vaccines, other observers made a far more
serious objection, namely, that the results of such tests merely demonstrated the antigenicity instead of the immunizing power of the vaccines examined. Since, however, in the experience of many workers a considerable parallelism existed between the results of agglutination tests and those obtained with active immunization of test animals, one should not be rash in denying the value of the former, far more expedient, method. It also deserves great attention that, as indicated by the studies of Burrows et al. (1947), possibly together with other antibodies O agglutinins do play a role in the protection against cholera infection.

It is generally acknowledged that the demonstration of bacteriolysins in the sera of specifically vaccinated experimental animals is indicative of a state of immunity against parenteral infection with V. cholerae. Since, however, a fundamental difference exists between the morbid process produced in this manner under experimental conditions and the disease spontaneously developing in man after the ingestion of cholera-contaminated materials, it is difficult to decide whether or to what extent the presence of bacteriolysins in the sera of cholera-vaccinated human subjects testifies to the existence of an immunity against natural infection with V. cholerae. Even some of the workers who were agreed that cholera vaccination is apt to protect man against such an infection, ascribed little importance to the presence of bacteriolysins in the sera of cholera-vaccinated human subjects. For instance, Papamarkou (1917) maintained in this connexion that these bodies may be absent or present at low titres only in the sera at times when the individuals in question are supposedly still protected against cholera infection by the previous vaccination.

However, while one must admit that the presence of bacteriolysins in the sera of cholera-vaccinated individuals furnishes no direct proof of the existence of an immunity against the infection, bactericidal tests with such sera are of value in so far as a considerable degree of parallelism has been found to exist between the results they yield and those of active and passive immunization tests. The technical difficulties attendant upon the bactericidal tests and their consequent tediousness render them less practicable in routine work than the above-evaluated agglutination tests. While, therefore, the latter seem to be preferable, it has to be kept in mind that no close parallelism has been found to exist between the results yielded respectively by these two serological methods. It also deserves attention that in the experience of Ahuja & Singh (1948) the outcome of bactericidal tests alone compared favourably with that of passive mouse-protection tests.

Complement-fixation tests with the sera of the vaccinated, while presenting considerable technical difficulties, seem to possess no superior value in comparison with the two serological methods discussed above.

(3) Active immunization tests. The method of testing cholera vaccines by actively immunizing experimental animals with the products under
examination and then challenging the animals thus protected with doses of *V. cholerae* found to be lethal for controls, which already guided Ferrán and Haffkine, has been continuously used by subsequent workers.

While at first exclusive use of guinea-pigs was made for this purpose, other species of experimental animals have been preferred by most of the recent workers. As noted before (see pages 317 and 318), Gohar & Isa (1948), in order to compare the efficacy of various cholera vaccines, resorted to active immunization tests with rats. White mice seem first to have been used for this purpose by Fennel (1919), to assess the efficacy of a cholera lipovaccine he had manufactured. However, large-scale use of these animals for the assay of cholera vaccines became possible only when Griffiths (1942) showed that suitably small yet highly virulent test doses for the intraperitoneal challenge of mice could be obtained by suspending the cholera vibrios chosen for this purpose in 5% mucin instead of in normal saline. It is of historical interest to note in this connexion the previous observation of Cantacuzène & Marie (1919a) that otherwise sublethal doses of *V. cholerae*, to which extracts from the small intestine or caecum of guinea-pigs had been added in small quantities, proved fatal if intraperitoneally administered to animals of this species.

Taking advantage of Griffiths' findings, the National Health Institute at Washington recommended tentatively in 1942 a mouse-protection test for the assay of cholera vaccines. As summarized by Ranta & Dolman (1943), this method

"involves vaccinating each of a group of at least 30 white mice, about five weeks old and weighing 8-10 gm., with a single intraperitoneal dose of the test vaccine, equivalent to about 400 million vibrios... An equal number of similar mice is set aside at the outset for control purposes. Fourteen days later one-half of the mice in both the vaccinated and non-vaccinated groups are given intraperitoneally approximately 500,000 live vibrios of a virulent Inaba-strain suspended in mucin, while the remainder receive similar doses of a virulent Ogawa type strain. The requirement is that at least 50 per cent of the mice in each vaccinated group should survive for 72 hours, while at least 75% of the non-vaccinated mice should die of cholera septicaemia within 72 hours."

Ranta & Dolman (1943) confirmed both the advantage of using suspensions of *V. cholerae* in 5% mucin for the challenge of immunized mice and the value of mouse-protection tests for the assay of cholera vaccines. They recommended in 1944 a modification of such tests, based upon the use of two spaced doses of the vaccines under examination and requiring survival of 100% of groups of not less than 15 mice challenged with 5 MLD of mucinized vibrios, and of at least 80% of batches of such animals challenged with 10 MLD. They considered it unnecessary to challenge the animals with both Inaba and Ogawa strains, as had been recommended by the National Institute of Health, because in their experience there existed a cross-protection between these two subtypes.

The problems involved in the mouse-protection tests were exhaustively studied by Burrows et al. (1947), who reached the conclusion that
A standard dose or fold increase method of titration of protective antibody was found to be impractical, but protective titer expressed as the ratio of the LD<sub>50</sub> dose for immune mice to that for control mice was reproducible within reasonable limits, and the results were comparable provided that the virulence of challenge strains was substantially the same. The variability was such that 100 fold differences in titer were regarded as significant, 10 to 100 fold suggestive, and 10 fold or less as not significant.

In a further study on the biological assay of cholera vaccines, Sokhey & Habbu (1950b) pointed out that the mouse-protection test suggested by Ranta & Dolman (1944) was not sufficiently exact because it used too large vaccine doses for the immunization of the animals. It was for this reason that Ranta & Dolman postulated the existence of a cross-protection between the Inaba and Ogawa subtypes which Sokhey & Habbu were unable to confirm.

The principle of a new method for the biological assay of cholera vaccines introduced by the two last-mentioned workers, which gave reproducible results within narrow limits, was to determine "the dose of vaccine required to protect 50% of the immunized animals against a challenge dose constant both in numbers and virulence and producing 100% mortality among the controls".

As noted before, Sokhey & Habbu preserved suitably virulent strains for challenging their animals by freeze-drying. After regeneration the selected strain was grown for three hours in nutrient broth and one part of a 10<sup>-5</sup> dilution of this was added to four parts of a 5% mucin suspension. 0.5 ml of this mixture, which was used for intraperitoneal injection of the test animals, contained about 100 000 organisms and represented 100 times the minimum lethal dose.

Though, as will be discussed below, some modern observers are inclined to place more reliance upon passive protection tests, there can be no doubt that properly conducted active immunization tests yield fully reliable results as far as the degree of immunity conferred by parenteral administration of the vaccines in question against parenteral infection with test doses of *V. cholerae* is concerned. It is clear, however, that the results of such tests, even if most favourable, furnish no direct answer to the question to what extent parenteral administration of the vaccines concerned is apt to protect man against oral cholera infection.

In order to obtain such final proof, it would be necessary to demonstrate that the vaccines in question, if subcutaneously administered, are capable of protecting the vaccinated animals or human subjects against oral cholera infection. As has been discussed before (see pages 321-322), experiments made in this respect by some earlier workers not only failed to furnish such final proof in a convincing manner, but gave as a rule frankly negative results. A few analogous trials made in man likewise proved disappointing. Metchnikoff (1911), summarizing the results of such attempts, stated that
in our 1893 memoir we referred to observations made in three persons, two of whom had been vaccinated by Haffkine, whereas the third served as control. All three showed the same symptoms of benign cholera which one observes in the majority of cholera infections in the laboratory. Ferrán himself as well as some of the individuals vaccinated by him suffered after ingestion of cholera vibrios from diarrhoea like the non-vaccinated. His co-worker Pauli had choleraic diarrhoea even though he had received 13 vaccine injections. Zlatogoroff (1904) . . . , though vaccinated four times with killed and living cholera vibrios, suffered after ingestion of a cholera culture from diarrhoea and was obliged to take calomel after he had a third fluid stool.” [Trans.]

It might be argued that the cholera attacks in the above-mentioned vaccinated subjects were invariably slight, but one must fully agree with Metchnikoff that the attempts to induce cholera artificially in man gave rather inconsistent results in non-vaccinated as well as in immunized individuals, producing often only slight symptoms, if any at all.

However, in marked contrast to the above-mentioned failures or uncertainties, recently Burrows & Ware (1953) obtained impressive results when immunizing guinea-pigs intraperitoneally with three doses of cholera O vaccine and afterwards challenging the animals by the intragastric route. As summarized by the two workers:

"Active immunization with cholera O vaccine results in a 14-fold increase in the ID₅₀ dose (median infective dose) at the height of the immune response, 4 days after a course of vaccine, which declines to 8.7-fold at 14 days and 1.9-fold at 28 days."

These results as well as previous observations of Burrows and co-workers on the appearance of antibodies in the faeces of actively cholera-immunized guinea-pigs and human volunteers, which will be discussed later, seem to endorse the value of active immunization tests for an assay of cholera vaccines.

(4) Passive protection tests. A passive mouse-protection test for assaying the results of cholera vaccination has been recommended by Griffiths (1944). As summarized by Burrows et al. (1947), this worker, when reporting the results of titration of protective antibodies in the sera of immunized human volunteers,

"expressed the titer in two ways, the number of LD₅₀ doses protected against by 0.1 ml of serum, and the amount of serum required to protect 50% of the mice receiving various doses of vibrios. By the first method, normal serum showed a titer of less than 3,000, and the immune serum of 100,000 to 200,000. By the second, 0.1 ml of normal serum did not protect against 590,000 vibrios, and of pooled immune serum, 0.068 ml protected 50% of mice receiving 59 million vibrios, 0.01 ml 50% of those receiving 5.9 million, and 0.0014 ml those receiving 590,000 vibrios."

Burrows et al. (1947), using the sera of 25 cholera-immunized volunteers for passive mouse-protection tests, formed a most unfavourable opinion on the value of this method. However, Ahuja & Singh (1948), using guinea-pigs for passive as well as for active protection tests, reached the conclusion that the former represented "the most sensitive method available in the
present state of our knowledge for demonstrating differences in the immunizing value of vibrio strains”.

As has been noted before, Ahuja & Singh considered bactericidal tests fairly trustworthy because they gave a response approximately parallel to the results of passive protection tests. They were, on the contrary, not favourably impressed by the utility of agglutination tests with the sera of vaccinated subjects for an assessment of the value of cholera immunization.

Mechanism of active cholera immunity

As pointed out by Pfeiffer & Wassermann (1893) in a classical study on the mechanism (Wesen) of the active immunity against cholera, the fact that immunized guinea-pigs resisted challenge with larger amounts of living cholera vibrios than non-immune animals, could be interpreted by assuming that “immunization might have conferred either antitoxic or bactericidal properties”.

Exhaustively investigating which of these two factors was at work, Pfeiffer & Wassermann found that guinea-pigs, regardless of whether they had been immunized with live or killed vibrios, by the subcutaneous or the intraperitoneal route, were practically as susceptible to intraperitoneal challenge with killed cholera vibrios as non-immune animals. It was clear, therefore, that the immunized animals had not acquired a resistance against the cholera toxin (Giftfestigkeit). On the other hand, it could be shown that living cholera vibrios injected into the peritoneal cavity of immunized guinea-pigs perished there far more rapidly than was the case in normal animals. Even if immunized guinea-pigs succumbed to challenge infection, because they had been injected with overwhelming doses of V. cholerae, their peritoneal cavity was as a rule sterile. The conclusion reached by Pfeiffer & Wassermann on account of these experiments as well as of passive immunization tests, which will be discussed later, was that

“it was erroneous to consider cholera immunity as a resistance against the toxin [Giftfestigung], as has been invariably done in the previous publications. In active as well as in passive immunization there develop exclusively bactericidal properties”. [Trans.]

An identical conclusion was reached independently by Sobernheim (1893) who stated that

“substances must have been produced in the blood of [cholera-] immunized animals which impede the development of living bacteria and, therefore, the production of the lethal amount of toxin, but which do not interfere with the deleterious action of the already formed toxin. Consequently, the animals are immune in the strict sense, but not resistant to the toxin [giftfest]!”. [Trans.]

As can be gathered from a study of the literature, particularly from a valuable summary by Hetsch (1912), the views of Pfeiffer & Wassermann and of Sobernheim, though soon adopted as the creed of the official German
Most noteworthy in this connexion are the views of Metchnikoff (1895) and some other French observers (see Hetsch, 1912), according to whom not a humoral immunity depending upon the action of bactericidal substances but the phagocytic activity of the leucocytes took the decisive part in the destruction of the cholera vibrios in the bodies of immunized animals. However, Pfeiffer (1894b), quoting experimental observations according to which vibriolysis took place in the peritoneal cavity of cholera-immunized guinea-pigs without any marked participation of leucocytes, maintained that phagocytosis, instead of being of prime importance in the process of cholera immunity, was an accompanying phenomenon (Begleiterscheinung) of a secondary character—an opinion which appears to have been shared by most subsequent workers.

Another noteworthy objection to the views of Pfeiffer and colleagues was made by Gruber (1896), in whose opinion agglutination of the causative organisms was of primary importance in the process of cholera immunity, the agglomerated vibrios then becoming amenable to the action of protective substances present in the bodies not only of immunized but also of normal animals.

However, as summarized by Sobernheim (1897) and by Hetsch (1912), Gruber’s theory was not in accord with the observations of several other workers. Pfeiffer & Kolle (1896) stressed in this connexion that the phenomenon of agglutination observable in vitro represented merely a passing stage, after which the vibrios again became capable of multiplication. More important still, they as well as other workers showed that cholera-immune sera, including the sera of convalescents, even though they had become devoid of agglutinating properties for various reasons, could still exert a bacteriolytic action in the animal body. It also deserved great attention that, as demonstrated for instance by Kolle (1901), different methods of immunization led to differences in the antibody content of the resulting sera, agglutinins appearing rapidly and to a high titre in the sera of intravenously immunized animals in particular, whereas subcutaneous or intraperitoneal administration of V. cholerae led to the production of prevalently bacteriolytic sera. Considering these and analogous experiences, Hetsch (1912) concluded that

“the specific bacteriolysins of R. Pfeiffer and the agglutinins of Gruber-Durham are different substances occurring side by side in the cholera-immune serum. The agglutinins can be considered as the result of a reaction of the organism to the infection and to some extent also as indicators of an immunity. The typical bacteriolysis, however, ... is produced solely through the bactericidal substances of R. Pfeiffer.” [Trans.]“

However, while feeling convinced of the paramount importance of the bacteriolysins in the protection of immunized guinea-pigs against infection with V. cholerae, Pfeiffer & Wassermann (1893) warned against using these
Theoretically interesting facts for an explanation of the immunity against human cholera, because as they stressed, the latter was absolutely different from the mixed process of infection and intoxication which is produced in guinea-pigs through intraperitoneal injection of cholera bacteria.

In order to study in which organs of the animal body the cholera-immune bodies were formed, Pfeiffer & Marx (1898) subcutaneously injected strong young rabbits with agar-slant doses of heat-killed cholera vibrios and then determined the bacteriolytic titres of their sera and leucocytes as well as of extracts of their organs, prepared by (a) triturating weighed quantities with the aid of glass powder; (b) mixing the triturates with measured amounts of broth; and (c) removing the glass particles by centrifugation after one day's storage in the refrigerator.

While obtaining no evidence that the leucocytes served as the matrix or even as the vehicles of the cholera immune bodies, Pfeiffer & Marx found, as summarized by Hetsch (1912), when examining after different intervals the extracts of the various organs for their antibody content that, hand in hand with a rapid increase of the immunity, in certain organs a considerably higher quantity of bacteriolysins was demonstrable than in the circulating blood. This held true in the first place of the spleen and the bone-marrow, next of the lymph-nodes and the lungs. Unexpectedly it was further found that in the majority of the experiments the spleen contained already during the second day after the vaccination clearly demonstrable amounts of cholera immune bodies, even when hardly any traces of such were recognizable in the blood serum.

In the opinion of Pfeiffer & Marx, the abundance (Plus) of the immune bodies found in the spleen, bone-marrow, and the lymph-nodes indicated that a rapid production of these substances took place there, which was in excess of their secretion into the blood-stream. Apparently this excess of immune bodies in the above-mentioned organs gradually decreased and was no more manifest when immunity had become maximal. While these findings referred in the first place to the bacteriolysins, Pfeiffer & Marx obtained some evidence to show that the agglutinins behaved in an identical manner.

On account of these and related observations Pfeiffer & Marx felt convinced that the production of cholera-immune bodies took place in the blood-forming (blutfbereitenden) organs, the spleen, the bone-marrow, and the lymph-nodes.

This conclusion of Pfeiffer & Marx, being in accord with the now generally accepted concept that the reticulo-endothelial cells, found in organs like the spleen, the bone-marrow, the lymph-nodes, and the liver, are the site of production of the immune bodies, continues to be considered valid. However, a considerable debate arose over the question whether a production of cholera-immune bodies took place in the intestine.
Attention has first to be drawn in the latter connexion to observations made by Cantacuzène (1894), Cantacuzène & Marie (1919a, 1919b), and Inouye (1928).

Cantacuzène (1894) noted that cholera vibrios which had been introduced by the intragastric route into subcutaneously or intraperitoneally vaccinated guinea-pigs disappeared from the small intestine after 3 hours, whereas they persisted abundantly for 24 hours in non-vaccinated animals. He postulated, therefore, that a "bactericidal milieu" existed in the small intestine of cholera-vaccinated guinea-pigs.

As already alluded to (page 333), Cantacuzène & Marie (1919a) found that extracts prepared from the small intestine of guinea-pigs by mincing, drying in vacuo, suspension in normal saline, storage in the refrigerator for 24-48 hours, centrifugation, filtration through paper and inactivation by exposure to 56°C for 1/2 hour, if added in quantities of 0.5 ml or 1 ml to a non-lethal dose of cholera vibrios, rendered the latter rapidly fatal for intraperitoneally infected guinea-pigs. This "activating" property was manifested not only by extracts obtained from the intestines of normal guinea-pigs, but to an even more marked degree by those derived from cholera-vaccinated animals. However, the extracts obtained from the latter category of animals were found to protect guinea-pigs against intraperitoneal injection with lethal cholera doses if administered subcutaneously 6 hours before infection.

Supplementing these observations by complement-fixation tests with intestinal extracts prepared in the manner described above, Cantacuzène & Marie (1919b) found that:

(a) The complement-fixing properties of extracts from the intestines of normal guinea-pigs were variable but as a rule not marked.

(b) On the contrary, the extracts obtained from the small intestines of guinea-pigs which had either received 24 hours previously a lethal dose of V. cholerae intragastrically, or had been injected intraperitoneally 24 or 72 hours previously with heat-killed cholera vibrios, showed most marked complement-fixing properties. The extract from the caecum of these animals gave much feebler reactions, their blood sera quite feeble reactions or even none at all.

(c) The extracts from the small intestines of solidly cholera-vaccinated guinea-pigs as well as their sera showed most marked complement-fixing properties.

Inouye (1928), working with extracts prepared like those of Cantacuzène & Marie, but usually without resorting to desiccation, found that:

(a) guinea-pigs which were intraperitoneally injected with intestinal extracts from animals vaccinated subcutaneously, resisted an immediately following challenge with lethal doses of V. cholerae, whereas guinea-pigs previously injected intraperitoneally with intestinal extracts from non-vaccinated animals succumbed even to non-lethal cholera doses;

(b) such non-lethal doses proved also fatal to animals which were given intraperitoneally extracts prepared from the liver or kidneys or muscles of normal guinea-pigs, in a solitary experience also to an animal injected intraperitoneally with the intestinal extract of an orally vaccinated guinea-pig;

(c) on the contrary, extracts from the liver or the spleen of parenterally vaccinated guinea-pigs did not exert a sensitizing action, thus not causing the death of animals challenged with sublethal doses of V. cholerae.

Though in view of the method of experimentation chosen by Inouye the value of his results appears to be limited, it is noteworthy that the extracts prepared from the intestines of parenterally vaccinated animals protected guinea-pigs against lethal cholera doses.
Cantacuzène (1920), discussing the findings made by him and Marie, felt convinced that the general immunity produced by parenteral cholera vaccination was accompanied, or rather preceded, by a rapidly appearing local immunity. Judging from ample and favourable experiences in the Romanian army during the Balkan wars and during the First World War, he considered parenteral cholera vaccination, which in his opinion conferred an antibacterial immunity, as an "absolutely rational" method.

As has been stated already, some of the advocates of oral cholera vaccination, such as Masaki (1922), maintained that this mode of immunization led to a local intestinal immunity, but one must fully agree with Hetsch (1928) that the evidence brought forward in this respect was by no means convincing, whereas not only other observations made in the case of cholera (see Wassermann & Sommerfeld, 1915, for example) but also ample evidence adduced in the case of other bacterial infections spoke against this concept. In order to support his view, Hetsch quoted the following conclusion reached by Neufeld (1924):

"There exist markedly different degrees of active immunity, but no different kinds, as if for instance through preliminary treatment with living cultures another kind of immunity would be produced as after the injection of killed cultures, or after oral administration of bacteria one different from that following subcutaneous administration or as if natural recovery from an (infectious) disease would necessarily [grundsiitzlich] lead to a state of immunity different from that following artificial immunization. The new knowledge on the formation of antibodies supports the basic concepts of Ehrlich, even though his ideas have to be modified in a few respects." [Trans.]

Attention has now to be paid to recent exhaustive studies by Burrows and his co-workers, which shed new light on the mechanism of active immunity against cholera.

Taking advantage of the new methods for the isolation and purification of the endotoxin of \textit{V. cholerae} introduced by Burrows (1944), he and his co-workers studied first the immunological properties of this endotoxin (see Burrows et al., 1944). The main results of this investigation were that:

\textit{(a)} An immunological, hapten-like activity of cholera endotoxin prepared by preliminary extraction with alcohol and three subsequent precipitations with chilled acetone was indicated by skin reactions in immune rabbits as well as by specific precipitation and complement-fixation produced by rabbit immune sera.

\textit{(b)} In a series of rabbit immunization tests with differently prepared types of endotoxin most remarkable results were obtained with dialysates, which in the case of animals immunized in 5 or 6 doses with a total of 7.5-8.5 mg led to a protective titre of at least 100,000 as well as to a marked agglutinin response (titres 1:50,000 or more).

Thus, as measured by the agglutinin response, these dialysates possessed antigenic properties markedly superior to those of whole vibrios. Similarly, mice could be actively immunized by three intraperitoneal inoculations of an alcohol-saline suspension of alcoholic toxin with doses far below those needed to afford the same degree of protection with heat-killed cholera vaccines.

\textit{(c)} It was not possible, however, to demonstrate an \textit{in vitro} neutralization of the activity of the endotoxin preparations by either antibacterial or anti-endotoxic sera or to
immunize mice either actively or passively against the lethal effect of intraperitoneal administration of the purified cholera endotoxin.

Studying the permeability of the small intestine of rabbits and guinea-pigs in vitro, Burrows, Wagner & Mather (1944) found that addition of living vibrios or of crude or purified cholera endotoxin to the Ringer-Locke solutions used for these tests markedly accelerated the rate of flow through strips of normal intestine. If, however, in place of these, strips of intestine from immune animals were used, they were completely, or almost completely, resistant to the action of the toxin and in its presence showed little or no difference in permeability to fluids from normal intestine in the absence of toxin.

As summarized by Burrows and his co-workers, these findings, which were consistent with and complementary to those recorded in the first paper by Burrows et al. (1944), indicated that "active immunity to Asiatic cholera in the experimental animal, and presumably also in man, includes antitoxic as well as antibacterial immunity".

It deserves attention, however, that according to further experiments recorded by Burrows (1953), the immunity resulting from active immunization with purified cholera endotoxin proved to be inferior to that produced by active immunization with O antigen.

Reporting upon observations made in guinea-pigs which had been infected with *V. cholerae* by the intragastric route following alkalinization and intraperitoneal administration of opium tincture, Burrows, Elliott & Havens (1947) stated that

(a) the infection thus produced was confined essentially to the lumen of the bowel with no consistent or significant spread into the tissues and organs, and could be considered to be of a true nature in view of an enormous multiplication of the vibrios in the intestine of the animals;

(b) prior administration of a non-lethal dose of *V. cholerae per os* or intraperitoneal immunization with 2 mg of cholera O vaccine two weeks before challenge protected the animals against as much as three lethal doses and altered the pattern of vibrio excretion characteristic of non-immunized infected animals by bringing about a sharp reduction in the number of organisms, especially early in the infection, and usually leading to a lessened persistence of the infection;

(c) antibody activity, manifested by the appearance of agglutinins and protective antibodies, and shown to be due to the presence of immune globulin, was demonstrated in the faeces of immunized animals and also in the faeces of human volunteers who had been vaccinated against cholera;

(d) the antibody in the faeces, called *coproantibody* to distinguish it from that in the serum, though appearing early and reaching peak titres before antibodies became manifest in the serum, disappeared in contrast to the serum antibodies in 3-4 weeks.

The correlation found to exist between the pattern of vibrio excretion characteristic for cholera-immunized animals and the presence of *coproantibody* led to the conclusion that "effective immunity to enteric infection is associated with pre-existing *coproantibody*."
As shown by further studies of Burrows & Havens (1948), immune globulin, immunologically indistinguishable from immune-serum globulin, was excreted in the faeces and urine of actively or passively cholera-immunized guinea-pigs as well as of cholera-vaccinated human volunteers. It was established in this connexion that the barrier between the tissues and the lumen of the bowel was readily permeable to the immune globulin, which thus could pass in either direction.

Burrows & Havens confirmed that there was a lag between the appearance of peak agglutinin titres in the faeces and urine of immunized guinea-pigs and in their sera respectively. This lag was still more conspicuous in cholera-vaccinated human subjects, in whom the peak of the faecal agglutinin titre was reached about two weeks after the second inoculation, and that in the urine apparently a few days later, as compared to the occurrence of peak titres in the serum after 30 to 42 days. There was also a difference in the disappearance of the antibodies, the agglutinins demonstrable in the urine and faeces falling to insignificant levels in the case of immunized guinea-pigs after 3–4 weeks, in that of human subjects two to three months after vaccination, whereas—as far as could be ascertained—the antibodies persisted in the sera for considerably longer periods.

It could not be established definitely where the faecal antibodies were formed. Considering various possibilities, Burrows & Havens stated that:

"It is possible that fecal antibody is that which is formed locally. The assumption that, with the general antigenic stimulus of parenteral inoculation, the antibody-forming cells of the intestine form antibody to excess much more rapidly than those of, for example, the spleen, seems unlikely and is without foundation. The corollary assumption, that the association of falling fecal titer and rising serum titer may be explained by an earlier cessation of antibody formation by the local cells or a breakdown of the mechanism of diffusion or secretion, is hardly tenable especially in view of the results of experiments reported here in which fecal titer was maintained by periodic reinoculation. Neither of these need be made, however, for it is possible that the observed independence of serum and fecal antibody titers is a concentration effect. Thus, the dilution of immune globulin liberated by antibody-forming cells during the early stages of immunization is relatively much greater in the body fluids than in the feces, but antibody accumulates in the tissues and not in the feces. Rising serum antibody would, then, represent a rate of accumulation of serum globulin while the titer of fecal antibody would reflect more directly its rate of diffusion from the antibody-forming cells."

The findings made in regard to the coproantibody were confirmed by some further investigations of Burrows and co-workers, particularly by the results of a study on the quantitative relationship between the faecal and serum antibodies made with the aid of complement-fixation tests by Koshland & Burrows (1950).

It would be rash to assert that the observations of Burrows and colleagues, the main features of which alone could receive attention within the scope of the present disquisition, have fully solved the problems of the mechanism of active cholera immunity. In fact, they have furnished reason
to assume that, as Burrows et al. (1947) put it when commenting upon the mouse-protection test:

"It may well be that protection is a manifestation of more than one kind of antibody, possibly O agglutinin, bacteriolysin, immune opsonin and the like, as well as antibody to the vibrio endotoxin; at least present evidence does not justify the assumption that protective antibody is homogeneous."

There can be no doubt, however, that the demonstration of a local response to parenteral cholera immunization in the intestine, manifested by the rapid appearance of coproantibody, goes a long way to support the assumption that this method of immunization is an effective means to protect man against natural infection with *V. cholerae*.

### Passive Immunity

Observations which demonstrated the possibility of conferring a passive immunity against cholera seem to have been recorded first in 1892 by Gamaleia, Klemperer and Lazarus.

Gamaleia reported at a meeting of the Société de Biologie, Paris, (October 29, 1892) that Ketscher in St. Petersburg had (a) found that the milk of cholera-immunized goats, if intraperitoneally administered to guinea-pigs in 5-ml doses, protected the latter animals against lethal doses of *V. cholerae* given intraperitoneally or intramuscularly, and (b) noted even the survival of guinea-pigs which had received intraperitoneal injections of such milk after they had been infected with cholera vibrios by the intramuscular or intraperitoneal route.

Klemperer (1892c), besides confirming that the milk of immunized goats was capable of protecting guinea-pigs against intraperitoneal cholera infection, also found the same to hold true of the serum of a human volunteer who had been injected with 5 ml of milk from a cholera-immunized goat. Klemperer (1892b) further established that the serum of persons vaccinated with living or heat-killed cholera vibrios protected guinea-pigs against intraperitoneal injections of *V. cholerae*.

As already alluded to, Lazarus (1892) found that the serum of cholera convalescents, if administered in extremely small doses (minimum one decimilligram, 0.0001 g) intraperitoneally to guinea-pigs, protected the animals against intraperitoneal challenge with lethal doses of *V. cholerae* made a few hours afterwards. He established, on the other hand, that even enormous doses of cholera convalescent serum were incapable of saving previously cholera-infected guinea-pigs which already showed signs of illness. Lazarus maintained, therefore, with great reason that

"in the case of animals which it was possible to save from death through treatment [with convalescent serum] following infection, we cannot speak of a successful cure but rather of an immunization administered during the incubation stage." [Trans.]

As can be gathered from a study of the literature (see summaries by Hetsch, 1912, and Kraus, 1929), during the years following these initial findings the question whether passive immunization against cholera conferred an antitoxic or merely a bactericidal immunity became the subject of considerable debate. Though, as will be shown later, this controversy is now of historical interest rather than of actual importance, the
following of the several claims made for the production of cholera-antitoxic sera (see in addition to the summaries just mentioned those of Hetsch, 1928, Harvey, 1929, and of Burrows et al., 1944) deserve attention.

Ransom (1895; see also Ransom & Kitashima, 1898) immunized goats and horses with a soluble cholera toxin, which he claimed to have obtained by heating 5- to 10-day-old broth cultures of *V. cholerae* for a short time at 100°C, filtering through a Pukal filter and then resorting to concentration *in vacuo* and alcohol precipitation at 30°C. As admitted by Ransom himself (1898), his sera possessed feeble (schwache) antitoxic properties. Pfeiffer (1895) even maintained that the antitoxic effects of Ransom's serum were not higher than those of normal sera.

Metchnikoff and co-workers (1896) prepared cholera sera by immunizing goats and horses as well as guinea-pigs and rabbits with what they considered a soluble toxin of *V. cholerae*. This thermostable product was obtained from strains the toxigenicity of which had been enhanced through passage in collodion bags kept in the peritoneal cavity of guinea-pigs. The vibrios in question were afterwards grown in a medium containing besides 2% peptone, 2% gelatin, and 1% sodium chloride, also 10% of normal horse serum for 4 days (when toxicity was found to be maximal) and then filtered.

The sera obtained with this toxic product through prolonged immunization of horses had marked bactericidal and agglutinating properties. At the same time they were endowed with what Salimbeni (1903) considered a somewhat feeble antitoxic power, at least 1 ml being needed to protect a guinea-pig against 4 lethal toxin doses. Nevertheless, as summarized by Salimbeni (1903), “the serum proved to be very efficacious prophylactically and gave in curative tests very good results in intestinal cholera of young rabbits produced experimentally according to Metchnikoff's method.”

Salimbeni (1908), continuing the above-described work, modified the methods originally adopted by Metchnikoff and collaborators (1896) by (a) ceasing to use the preliminary passage of the strains in collodion bags through guinea-pigs, which was found to be unnecessary when freshly isolated human cholera strains, never passed through animals and kept with rare transplantations on agar at room temperature, were available; (b) increasing the content of horse serum in the medium used for toxin production to 25%; and (c) adopting in place of the subcutaneous the intravenous route of immunization. He noted in this connexion that, while 1.5 ml of serum from a subcutaneously immunized horse had been needed to neutralize 4 lethal doses of cholera toxin, after continued intravenous immunization of the same horse 1.3 ml of its serum sufficed for this purpose.

It is noteworthy that prolonged intravenous immunization of a horse with living cholera vibrios (saline suspensions of agar cultures) also gave a serum which combined marked agglutinating and bactericidal properties with a quite marked power to neutralize the toxin. This was in contrast to the results of intraperitoneal immunization with living cholera vibrios attempted by Metchnikoff and collaborators (1896), the serum obtained in this manner from a horse, though endowed with marked agglutinating and bactericidal properties, showing less antitoxic power, since 1.5 ml were necessary to neutralize two lethal toxin doses.

As alluded to before (page 317), Macfadyen resorted, in order to liberate the cholera endotoxin, to the method of fragmentating growths of *V. cholerae*, obtained through cultivation on agar for 18 hours, at the temperature of liquid air. The thermostable endotoxic products obtained in this manner were capable of producing sera which possessed, besides agglutinating and bacteriolytic properties, anti-endotoxic power. For instance, the serum of an intravenously immunized goat was found to neutralize in guinea-pig tests 8 lethal endotoxin doses, whereas normal goat serum proved incapable of protecting these animals even against two lethal doses.

Brau & Denier (1906), in order to obtain toxic products for the manufacture of cholera-immune sera, (a) grew a strain of *V. cholerae* isolated in Saigon, which was found to coa-
gulate milk rapidly but was non-haemolytic, in a medium consisting of 90\% normal horse serum and 10\% defibrinated horse blood, and \( b \) resorted after an incubation at 39°C for 7 days to filtration first through paper and then through Chamberland or Berkefeld filters.

Summarizing their experiences when using sera produced with the aid of this toxic filtrate and, for the sake of comparison, also a serum raised by Salimbeni against live cholera vibrios, Brau & Denier stated that:

\( a \) Subcutaneous injection of the toxin into goats, rabbits, guinea-pigs and horses produces with difficulty an active immunity. The serum thus obtained is feebly antitoxic.

\( b \) Intravenous injection on the contrary immunizes the animals and leads to the appearance of very manifest antitoxic properties in their serum.

\( c \) The animals which had been intravenously injected with living cultures furnish a more active serum than those treated with the soluble toxins." [Trans.]

Brau & Denier maintained, therefore, with much reason that there seemed no need to establish a difference between the cholera toxin contained in the bodies of the microbes and that obtained in the culture fluid.”

It is important to add that \( a \) the serum produced by Brau & Denier had also quite good agglutinating properties (titre 1: 5000); \( b \) their toxin, the production of which they ascribed to a maceration of the vibrios in the course of cultivation, was found to be thermostable, a serum raised with a filtrate which had been heated for 20 minutes at 100°C proving as efficacious as those prepared with unheated toxic filtrates, and \( c \) Brau & Denier established that neutralization of lethal doses of their toxin by graduated serum doses did not take place according to the law of multiple proportions, as is characteristic of true bacterial exotoxins.

Kraus, summarizing in 1909 the results obtained in the production of antitoxic cholera sera (see Kraus, 1907; Kraus & Russ, 1908), stated “that not only the cholera vibrio, but also the El Tor vibrios and many other vibrios have the property to produce toxins (Gifte vom Charakter der als Toxine charakterisierten Gifte) and that these can be neutralized with specific antitoxins.” Therefore, in order to produce antitoxic sera, Kraus and Kraus & Russ used not only true \( V. \) cholerae strains, but also El Tor strains as well as the \( V. \) Nasik, a haemolytic vibrio not reacted upon by cholera-immune sera.

The sera raised with the toxins, i.e., the filtrates of 6- to 8-day-old broth cultures, of true cholera vibrios, though also producing bacteriolysis of El Tor vibrios, exerted an antitoxic action only against the \( V. \) cholerae toxins. The El Tor serum, while bacteriolytic not only for the El Tor but also for classical cholera vibrios, was found to be antitoxic not only in the case of these two organisms, but also of cholera-like vibrios. Serum obtained with \( V. \) Nasik toxins, while bacteriolytic for this organism only, proved antitoxic also for El Tor vibrios.

While finding guinea-pigs rather unsuitable for tests with these sera (see below), Kraus reported that:

\( 1 \) Mice which are infected with cholera vibrios are cured if one treats them either with antitoxic cholera serum or with serum obtained with the toxin of specific El Tor vibrios (i.e., El Tor vibrios in the strict sense). Sera obtained with \( V. \) Nasik toxins were ineffectual in this case.

\( 2 \) Mice infected with specific El Tor vibrios are cured if treated with sera prepared with El Tor or Nasik toxins. Cholera serum produced in horses raised completely [but, as stated by Kraus in a footnote, a cholera serum produced by Pfeiffer in goats neutralized also El Tor toxin].

\( 3 \) Mice infected with \( V. \) Nasik are cured if they are treated with sera prepared with El Tor or Nasik toxin. Cholera serum exerts no curative action.” [Trans.]
On account of these findings Kraus stressed that success in cholera treatment could be obtained only with those sera which contained antitoxins as well as bacteriolysins. It has to be noted, however, that the mice used in such tests could be saved only if treated with the appropriate sera not later than one hour after infection. Guinea-pigs could be saved but occasionally if given large serum doses intravenously half an hour after infection. Kraus warned, therefore, against concluding from favourable results obtained in one animal species upon the possible effect of the sera in other species and expressed at the same time the fear that man might react in respect to specific cholera treatment like the guinea-pig and not like the mouse.

Schurupow (1909), reporting upon tests with one of Kraus' cholera sera, which had been sent to Russia, stated that this serum possessed but minimal antitoxic properties and produced agglutination of cholera vibrios only at a titre of 1:500.

In order to prepare a cholera serum of his own, Schurupow tried first to immunize horses with living cholera vibrios (route not stated). However, the serum of these animals was found to possess only marked agglutinating and bactericidal properties but no antitoxic power. Schurupow admitted, however, that these experiences were not in accord with those of some other workers, Kraus for instance having obtained through subcutaneous immunization of horses with living cholera vibrios a serum, 0.07-0.1 ml of which neutralized 1 ml of toxin.

In view of the disappointing results he had obtained with live cholera vibrios, Schurupow immunized horses with Chamberland-candle filtrates of *V. cholerae* cultures which had been treated with alkali. The resulting sera possessed agglutinating properties (titre 1: 10,000), but had according to Schurupow no bactericidal power. As far as can be gathered from the somewhat scanty data furnished by this worker, his sera showed quite considerable protective and even curative action against the endotoxin, but unless quite high doses were administered prophylactically, the guinea-pigs tested showed marked signs of intoxication before eventually recovering.

As will be gathered from the above summary, the various workers enumerated, though using different antigens for the immunization of their animals, unanimously stated that they had obtained sera more or less endowed with antitoxic properties. The validity of these claims was, however, vigorously opposed by Pfeiffer and his co-workers.

Pfeiffer & Wassermann (1893), making passive immunization tests in guinea-pigs with cholera convalescent sera, found ample evidence of a bactericidal action of these sera, but none of a role of antitoxins, and concluded, therefore, that passive as well as active cholera immunity depended upon the presence of bacteriolysins. This contention was supported by Issaeff (1894) who found that the sera of cholera-immunized guinea-pigs as well as those of human convalescents, though endowed with marked protective and to some extent even with curative properties, possessed no antitoxic properties, the maximal cholera toxin doses tolerated by immunized guinea-pigs being not higher than those for control animals.

The findings of Issaeff were confirmed through exhaustive studies made by Pfeiffer (1895b) with the sera of goats which had been subcutaneously immunized for prolonged periods with increasing and finally with enormous doses of living cholera vibrios. Even the most potent of these sera were found to exert "no true antitoxic effect" either against the toxin of chloroform-killed cholera vibrios or against that contained in broth cultures of
V. cholerae which had been sterilized with toluene after an incubation for 20 days.

It seemed at first glance in contrast to these observations that guinea-pigs which had been injected intraperitoneally with a mixture of the toxic substances and large amounts of the immune serum tolerated toxin doses two to three times in excess of those lethal for the controls. Pfeiffer found, however, that almost as high a tolerance for increased toxin doses could be produced if, instead of immune serum, the serum of normal goats was used for such tests. He assumed, therefore, that under the conditions of these experiments the immune as well as the normal serum exerted merely an unspecific action by hampering and retarding the absorption of the cholera toxins.

A further interesting study of the problem presently under review was made by Pfeiffer & Friedberger (1908a) who used for this purpose El Tor sera put at their disposal by Kraus in comparison to a purely bactericidal cholera serum obtained through single intravenous injections of rabbits with minimal doses of V. cholerae. The El Tor toxin used to test these sera was obtained by (a) centrifuging the peritoneal exudate of guinea-pigs which had succumbed to intraperitoneal infection with V. El Tor, and (b) adding to the supernatant small amounts of the bactericidal cholera serum so as to lyse the organisms which had not become sedimented.

In confirmation of the experiences of Kraus and Kraus & Russ, Pfeiffer & Friedberger found that the acute effects of the thermolabile exotoxin produced by V. El Tor were neutralized by El Tor serum. They stated, however, that

"in the case of the true toxins and antitoxins we find an exactly quantitative relationship; the lethal dose is either neutralized or not. In the case of the mixtures of Tor exudates with Tor serum things are not so simple. Here one observes, even when manifold multiples of the protective minimal [serum] dose are used, a drop of the temperature [of the test guinea-pigs] down to 34°C, which may be accompanied by other signs of intoxication (most marked prostration) and which appears characteristically only 5-6 hours after injection of the mixture." [Trans.]

Pfeiffer & Friedberger concluded from these observations that the peritoneal exudates of El-Tor-infected guinea-pigs contained two kinds of toxins—a neutralizable exotoxin and a second component, which represented the endotoxin liberated in the peritoneal cavity of the animals through disintegration of the organisms, and which, as shown by a large series of carefully conducted experiments, was not neutralized by the El Tor sera. Pfeiffer & Friedberger further adduced evidence to show

"that in El Tor infection the antitoxic serum acted mainly through its bactericidal component, whereas the antitoxin contained in the serum according to our quantitatively conducted experiments was incapable of exerting a favourable influence on the course of the infection." [Trans.]
Preventive and curative tests made with the El Tor sera in cholera-infected guinea-pigs convinced the two workers that

"the El Tor antitoxin is by no means a universal antitoxin as is postulated by Kraus & Russ. It fails completely as far as the endotoxins of the cholera vibrios and also as far as the toxic substances are concerned which form in the body of cholera-infected guinea-pigs." [Trans.]

Entertaining no doubt that in the case of cholera as well as in that of El Tor infection the immune sera exerted only a bactericidal effect, Pfeiffer & Friedberger concluded that "so far the investigations of Kraus had furnished no proof for the existence of true antitoxins against the toxin of V. cholerae".

The views of Pfeiffer & Friedberger were fully shared by Raskin (1909) who, retesting Schurupow's serum found that this product, while exerting no anti-endotoxic action, possessed a high bacteriolytic titre. In accordance with these observations Raskin came to the conclusion that the curative action of Schurupow's serum in guinea-pigs was not superior to that shown by purely bacteriolytic sera.

Pottevin (1913b) found that a classical strain of V. cholerae, which had been isolated in Constantinople, possessed only a thermostable endotoxin which was neutralized neither by its homologous serum, produced through prolonged subcutaneous immunization of a donkey, nor by the serum raised in an identical manner with one of the two El Tor strains examined at the same time. The latter two strains possessed also an exotoxin which (a) was thermolabile; (b) was endowed with haemolytic properties; (c) in contrast to the endotoxin of the cholera and El Tor vibrios was lethal for intravenously injected pigeons: and (d) also in contrast to this endotoxin was neutralized within certain limits by either of the two above-mentioned donkey sera.

These interesting observations, besides confirming the views held by Pfeiffer in regard to the toxin of V. cholerae, also speak in favour of an identity of the El Tor exotoxin with the "haemotoxin" (haemolysin) of these organisms. The continued presence of endotoxic properties after the action of the exotoxin had been inhibited by heating, as demonstrated by Pottevin, was probably responsible for the belief of some workers that the V. El Tor produced a soluble toxin distinct from its haemolysin.

Carrière & Tomarkin (1910) summarized the results of an exhaustive experimental study on the problem of cholera serotherapy, made in Kolle's laboratory at Berne, Switzerland, as follows:

"1) Bacteriolytic or purely bactericidal cholera sera obtained through a few intravenous injections of cholera vibrios possess the least curative effect, even when their lytic and agglutinating titres are high.

"2) The greatest curative effect in animal experiments is exerted by sera which have been produced through prolonged immunization with cholera bacteria, particularly if a mixture of the sera of different species of animals, immunized ... by various methods is used."
PROBLEMS IN IMMUNOLOGY

3) Such sera contain considerable amounts of anti-endotoxins, but it is difficult to assess the anti-endotoxic properties in view of the comparatively low neutralizing power of the sera and the varying resistance of animals to the cholera toxins.

4) The law of multiples is not valid for the anti-endotoxins of the cholera serum.

5) The administration of comparatively large amounts of bactericidal serum is innocuous for animals with experimentally produced cholera peritonitis, provided that considerable amounts of anti-endotoxic substances are administered simultaneously.

6) Serum treatment of cholera patients is innocuous, if one uses, instead of purely bactericidal sera, sera with some anti-endotoxin content. In contrast to the widely accepted opinion that owing to the action of the bacteriolysins serum administration leads to the massive liberation of endotoxins, our sera as well as those manufactured in Russia caused absolutely no harm even if given to cholera patients in very large doses.

7) To obtain therapeutic success, the administration of considerable amounts of serum is indispensable.

8) It is essential to use for therapeutic purposes exclusively those sera which have been obtained through immunization of different animals prolonged as much as possible and of different animal species by subcutaneous and intravenous administration of living and killed cholera vibrios as well as of endotoxins and extracts of the latter.

Commenting upon the results of their studies, made mainly with the sera of goats, horses, and rabbits, Carrière & Tomarkin found no reason to abandon the concept of the cardinal importance of the cholera endotoxins, but argued against the opinion often advocated that it was impossible to produce antitoxins against them. However, while claiming to have proved the fallacy of such beliefs, Carrière & Tomarkin admitted that the endotoxic substances at work probably do not represent the whole endotoxin of the bacterial cells in its original form and produce antibodies which do not completely correspond to the endogenous bacterial toxins. The incomplete efficacy of the sera might be due, therefore, not to the fundamental impossibility to produce anti-endotoxic substances, but merely to the inadequacy of the methods available for immunization.

Kolle (1909a), in a preliminary communication on the work of Carrière & Tomarkin, expressed full agreement with the views of these two workers and maintained that prolonged immunization leads, perhaps more in some animal species than in others, to the formation not only of bacteriolysins and agglutinins but to some extent also of anti-endotoxins and, further, of complement-fixing substances and bacteriotropins. Moreover, this enumeration probably does not exhaust the substances which are involved in the treatment of cholera. It would be wrong, theoretically, to dismember an immune serum and to conclude from such theoretical considerations upon its efficacy in animal experiments and in patients.

However, while one must agree with Kolle that the mechanism of passive cholera immunity is of a more complex nature than is indicated by the rigid dicta of Pfeiffer and his school, and that anti-endotoxins probably play a role in it, one should not lose sight of the fact that all cholera sera produced thus far exhibited but feeble anti-endotoxic properties, if any at all. It is under these circumstances not surprising that, as
CHOLERA

will be further discussed in Chapter 9, in spite of the optimistic opinions expressed by some of the early observers, the inefficacy of cholera serotherapy is now generally admitted (see Hetsch, 1928, and particularly Kraus, 1929). In fact, apart from a solitary attempt made by Ghosh (1935, 1936) the method of treating cholera patients with the aid of immune sera has not been used any more.

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