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EXPERT COMMITTEE ON
RESPIRATORY VIRUS DISEASES

First Report

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GENEVA
1959
EXPERT COMMITTEE ON RESPIRATORY VIRUS DISEASES

Stockholm, 11-15 August 1958

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EXPERT COMMITTEE ON
RESPIRATORY VIRUS DISEASES

First Report *

The Expert Committee on Respiratory Virus Diseases met in Stockholm from 11 to 15 August 1958. This Committee took over the responsibilities of the Expert Committee on Influenza, which met in 1952, and in addition considered present knowledge of other respiratory diseases caused by viruses.

The Committee was greeted by Dr M. Tottie, representing the Royal Medical Board of Sweden, and the session was opened by the Secretary on behalf of the Director-General of the World Health Organization. Dr C. H. Andrewes was elected Chairman; Professor V. M. Zhdanov, Vice-Chairman; and Dr M. R. Hilleman, Rapporteur.

During the meeting a joint session was held with the Joint WHO/FAO Expert Committee on Zoonoses (see section 2.3.2, page 11).

1. GENERAL REVIEW OF RESPIRATORY SYNDROMES AND RESPIRATORY VIRUSES

From the public health standpoint, the various diseases of the human respiratory tract caused by viruses—diseases often associated with bacterial complications—constitute an immense and still unsolved problem. They range from trivial illnesses to rapidly fatal conditions. They cause sporadic cases, localized outbreaks and explosive epidemics. Though often regarded as being more of a nuisance than a threat, they nevertheless are of great economic and medical importance, and in all countries are a major cause of morbidity.

In the past, these diseases have been classified by the clinician largely on clinical and anatomical grounds, though distinctive epidemiological

* The Executive Board, at its twenty-third session, adopted the following resolution:
  The Executive Board
  1. NOTES the first report of the Expert Committee on Respiratory Virus Diseases;
  2. THANKS the members of the Committee for their work; and
  3. AUTHORIZES publication of the report.
1 Wild Hth Org. techn. Rep. Ser., 1955, 64

— 3 —
features have led to the separation of syndromes such as that of influenza. As knowledge grows concerning the viruses found in association with the respiratory tract, it becomes increasingly clear that differential diagnosis requires the aid of the laboratory if it is to be based soundly upon etiology. Nevertheless, much of the knowledge is too recent for the clinical significance of the various viruses to have become established. For the present, therefore, the classification recommended by the Committee for the various respiratory syndromes is extremely simple. It is urged that in the future care should be taken to avoid the designation of new syndromes until knowledge concerning the etiological virus or viruses has advanced to the stage where the condition can be recognized as an entity.

There are good historical and clinical grounds for the sub-division of the respiratory syndromes caused by viruses into four groups. These are:

1. Influenza—caused by the influenza viruses types A, B and C.

2. Common cold—of which the causal virus or viruses are as yet unknown but which can be transmitted by bacteria-free filtrates of nasal secretions.

3. Atypical or virus pneumonia—known to be caused by several virus and rickettsial agents and separable on clinical, radiological and therapeutic grounds from bacterial pneumonia. Agents thus far identified are psittacosis (ornithosis) virus, Coxiella burnetii of Q fever, adenoviruses (see section 3, page 26) and the virus first described by Eaton, Meiklejohn and van Herick (see section 4.3, page 31).

4. Unclassified acute respiratory disease (febrile catarrh) (see section 4). This group undoubtedly includes several different pathological entities which, however, may overlap, and the illnesses in individual patients are often best described by the clinician in terms of the anatomical area which is affected. Nevertheless, some syndromes have already been described, and these include:

   a. Acute respiratory disease, resembling influenza but presenting some contrasting features—sometimes associated with adenovirus infection.

   b. Non-bacterial exudative pharyngitis—often associated with adenovirus infection.

   c. Pharyngo-conjunctival fever—associated with adenovirus infection.

   d. Kerato-conjunctivitis—adenoviruses have been identified. (This syndrome has been included here, although not strictly a respiratory infection, since it is one form of adenovirus infection.)

   e. Obstructive laryngo-tracheo-bronchitis of infants (croup)—croup-associated (CA) virus, influenza, and possibly other viruses.
Acute, often fatal, pneumonia in infants—Sendai virus (haemagglutinating virus of Japan), adenoviruses and probably other viruses.

The problems of respiratory diseases might be resolved by the following steps:

(1) Discovery of the etiological agents and the establishment of their relationship to human illness.

(2) Development of suitable and reliable laboratory diagnostic procedures and techniques.

(3) Definition of the clinical features and syndromes to which the etiological agents are related, giving special attention to severity and mortality.

(4) Definition of attack-rates for the specific disease according to geography, age, occupation, and other considerations.

(5) Development and evaluation of specific preventive measures, including vaccines against agents of major importance.

If an understanding of the significance of all the various known respiratory viruses is to be achieved, it is essential that reliable information be collected from as many countries as possible. It is here that the services of the World Health Organization could do much to promote and foster new developments. The greatest obstacle to the individual laboratory working in this field is the complexity and expense of the material needed to study so many new viruses. WHO should be encouraged to establish international centres for the identification and typing of new virus isolates. In addition, its offices might be used to obtain and distribute the reagents and materials needed for specific laboratory diagnostic tests. Finally, WHO might function as a central clearing-house for the receipt and dissemination of information concerning respiratory illnesses of miscellaneous etiology along the lines at present employed for epidemic influenza.

The subsequent sections of this report are devoted to a description of the known viruses of respiratory disease and to a summary of the present knowledge relating to them. Sufficient knowledge is at present available concerning the influenza viruses and the adenoviruses to warrant special consideration in separate sections. The remaining agents are grouped under the single heading of miscellaneous respiratory viruses. Although much information is at present available concerning psittacosis and Q fever, these diseases were not considered in detail by this Committee because of the more extensive discussion by the Joint WHO/FAO Expert Committee on Zoonoses.¹

2. INFLUENZA

2.1 Evolution and grouping of influenza viruses

The report of the Expert Committee on Influenza noted the existence of three antigenic types of human influenza virus, known as types A, B and C. It emphasized the antigenic lability of type A and, to a lesser extent, of type B virus, and noted the importance of such antigenic variation in relation to the problem of classifying viral strains and of immunizing against the disease.

In discussions concerning antigenic variation among influenza viruses and its effect upon the epidemiology of the disease, note must be made of the two kinds of antigens present in influenza virus. The first is the "soluble" antigen, which is readily measured serologically by the complement-fixation test (see section 2.9, page 23). One "soluble" antigen is common to all sub-groups and strains of influenza A, and another different one is common to all strains of influenza B. There is no antigenic crossing between types, and this "soluble" component appears to bear no relationship to immunity from the disease. The second kind of antigen is the haemagglutinins of the viruses which appear to be concerned directly in immunity. The composition of the virus in relation to these haemagglutinins accounts for the antigenic differences between strains within a given type and also for the antigenic variability which influenza virus manifests. Various techniques may be employed for measuring the specific antigens of influenza virus, and these include haemagglutination, complement fixation (washed whole-virus or separated haemagglutinating component) and other methods.

The antigenic changes which have occurred among the influenza A viruses of human origin since the first virus was recovered in 1933 have consisted generally of minor shifts from year to year, punctuated occasionally, at intervals of a decade or more, by the appearance of strains of markedly different antigenic form which are predominant thereafter. The first major change of this kind was observed to occur in 1946-47, when the A1 virus—often called influenza A-prime (see below)—first appeared, and influenza vaccine, consisting of strains of previous occurrence, afforded no significant protective effect. The second and most recent change was in February 1957, when the A2 or Asian influenza virus appeared. The latter change was considerable and represents the most abrupt shift in the recorded history of influenza viruses, coinciding with the appearance of a classical pandemic. The influenza B viruses have also been observed to undergo change in antigenic composition, but the alterations have been less sharply defined than the major shifts which have been noted for influenza A viruses. Influenza C viruses have shown no detectable variation in antigenic constitution.
The report of the Committee on Influenza, recognizing the limitations in knowledge of influenza viruses of proven human origin, tentatively divided the type A strains into three main groups—namely, WS (1933), PR8 (1934) and FM1 (1947)—according to their prototype strains. Newer knowledge and more complete information relating to the antigenic constitution of these agents warrants recognition at this time of three principal sub-groups or families of type A virus. These are:

1. **Sub-group A**. This includes both the former WS and PR8 groups and those A viruses predominant during the period from 1933, or earlier, up to 1946.

2. **Sub-group A1**. This sub-group, formerly designated FM1 (1947) and commonly referred to as A-prime, represents those strains which were predominant from 1946 to the time of appearance of the Asian influenza virus in 1957. This sub-group continued to appear early in 1957 and may continue to occur in the near future.

3. **Sub-group A2**. This is the new Asian sub-group and is exemplified by the A2/Japan/305/57 and the A2/Singapore/1/57 strains. This seems likely to be the dominant sub-group in the next few years.

In the earlier report, the influenza B viruses were divided into two groups with the general characteristics of the Lee (1940) and Bon (1943) strains. Since then, further antigenic changes have occurred, so that vaccines prepared with B-Lee (1940) virus were ineffective against the influenza B strains of 1954. The Committee wishes to record the continued antigenic alteration of influenza B viruses but makes no recommendation at present concerning the designation of sub-groups.

The Committee wishes also to recommend strongly the uniform employment of the code system for designating newly-isolated influenza virus strains. In the example A2/Japan/305/57, A refers to the type, 2 to the sub-group or family, Japan to the place of origin, 305 to the strain serial number and 57 to the year of isolation. The number referring to the sub-group may be included or omitted. Individual laboratories are asked not to designate new sub-groups without consultation with the international reference centres. The allocation of blocks of serial numbers will continue to be made by the World Influenza Centre in London and by the International Influenza Center of the Americas at the Communicable Diseases Center in Montgomery, Ala., USA.

### 2.2 Possible mechanisms for antigenic variation among influenza viruses

The mechanism for the antigenic changes which occur with influenza A and B viruses seems to reside in the ability of the virus to vary antigenically, coupled with the driving force of selection imposed by the necessity for the virus to survive in the partially immune host population. Thus, as
the human population gains in experience with a particular antigenic form of influenza virus, the general level of immunity is raised and it becomes more and more difficult for the virus to make effective contact with new susceptibles. Those viruses which are least influenced by the prevalent antibody will have the best chance for survival, host immunity providing the mechanism for selection. However, from time to time a new and greatly altered virus variant appears which may become predominant during the ensuing period.

The influenza virus particle contains many specific antigenic components which may vary both qualitatively and quantitatively. At least three distinct possibilities exist for the origin of the antigenic changes which occur. The first of these is related to the antigenic variation which might be expected to occur in any virus population. In this circumstance, the driving forces of variation and immunological selection may cause alteration in the relative preponderance of existing antigenic components, so that antigens which were important in strains of a previous period and have been relegated to a minor position may re-emerge to prominence. Support for this concept is given by the observation that most viral strains contain, in common, at least some of the specific antigenic components referable to the type but vary considerably in their content of the major components. Indirect support is provided by the apparent antigenic stability of type A swine influenza virus which may be related to the absence of a mechanism for immunological selection consequent on the relatively brief life-span of pigs. Finally, it has been shown in several laboratories that sera collected during early 1957 from aged persons who lived during the 1889-90 pandemic have a significantly greater amount of antibody against Asian influenza virus than have sera from younger persons. This observation has been interpreted to suggest that a virus which was prevalent during the last decade of the 19th century possessed an antigen or antigens in common with the Asian sub-group. Some suggest that this phenomenon may be related more to repeated experience with a multiplicity of influenza strains, resulting in a broader antibody pattern. Opposing the latter argument is the fact that in the oldest age-groups, the titres against Asian virus were often higher than against recent A and A1 viruses and could be disproportionately enhanced by vaccination as compared with other age-groups; such a result would not be expected if this antibody were due to repeated stimulation with a variety of minor antigenic components.

The second possible source for antigenic change relates to a viral mutation, involving the appearance of antigens not previously present. Such mutation may involve only a single antigenic component.

The third possibility lies in a hypothetical human or animal reservoir of virus which might or might not have been prevalent in man in the past. Proof of this hypothesis must await demonstration of the virus in such a habitat.
The question of whether the limits of influenza virus variation are small or large is of great importance, since upon it hinges the feasibility of preparing a single vaccine which will protect against all strains of virus. If all the important antigens of influenza virus could be included in a vaccine, it would be possible, theoretically, to provide a significant immunity against all the possible antigen combinations. However, the forces of mutation and selection might defeat this objective by causing the appearance of de novo of antigens which had not occurred previously. Faced with a situation such as arose with Asian influenza, it remains necessary, and becomes increasingly important, to maintain a constant watch on the antigenic constitution of influenza strains as they occur and to incorporate the new and different ones in vaccines as appropriate. The efforts of the world-wide network of WHO Influenza Centres should be continued and increased. World-wide co-operation among all nations is essential to this end.

2.3 Epidemiology

2.3.1 The 1957 pandemic

The pandemic of influenza in 1957, caused by the Asian virus, has been described by several authors. The virus was first detected in February 1957 in the Kwei-Chow province of China and from thence it spread to all continents during the next six months. The path of its progress was followed with unusual clarity by laboratory workers all over the world, largely because of the distinctive antigen of the A2 virus. Epidemiological, clinical and virus studies were made in many countries and will form the basis of future publications. The information available to the Committee suggested that the experience was in general uniform, but that some differences existed as between different countries, particularly in regard to the speed of development and the form of outbreaks. Four phases could be discerned in the involvement of the populations of particular countries. These were:

2.3.1.1 Introduction of the virus

The virus was spread by human contact, frequently through the medium of shipping and less often by aircraft. Outbreaks occurred chronologically in a manner which suggested the relatively greater importance of seaborne traffic as compared with air travel. Thus Cairo, which is the centre of extensive air transport from the East, experienced an outbreak no earlier than did adjacent countries. Ships undoubtedly were concerned in the arrival of the virus in the Netherlands and possibly on the east and west coasts of the USA, but the relative importance of air travel was at times impossible to assess. In the case of contiguous land masses, such as China and the USSR, spread occurred in relation to railways, such as the Trans-Siberian railway. In Israel, where the epidemic occurred some weeks after
the outbreaks in adjacent States, it seems likely that lack of direct traffic to and from these areas was responsible for the delay.

2.3.1.2 Phase of virus seeding

In many tropical countries and also in Japan, once the virus was introduced there appeared to be an immediate spread of infection accompanied by a rapid build-up of outbreaks into a general epidemic. But in other countries, and particularly in Europe and the USA, six or more weeks' delay occurred after the first detected entry of virus before a general outbreak involved large sections of the population. During this delay seeding of virus was occurring throughout the population. This process was sometimes silent, sometimes accompanied by local outbreaks, and sometimes detected by a chain of infection. Dispersion of travellers from conferences, scout jamborees or youth festivals assisted the spread of virus.

The factors chiefly concerned in the determination of the length of this phase of the pre-epidemic infiltration of the population are not known. Climate, social customs and the state of the schools appeared to be important, and it is possible that the virus was not always detected at the time of its first introduction. Schools were often closed for holidays in countries where a lag was experienced and the general epidemic coincided with, or quickly followed, their opening.

2.3.1.3 The main epidemic phase

In all countries the patterns of morbidity of the general epidemics were remarkably similar; attack-rates were high and outbreaks were frequently explosive, but the mortality was low. The age-specific attack-rates were also the same in most areas where information could be obtained. School-age children were heavily attacked, while adults, particularly those aged 40 or more, were relatively spared. Mortality, however, increased with age, although in some countries—Chile and the Philippines, for example—there was a high mortality also among infants. The pattern of mortality in England and Wales showed a greater proportion of deaths in the younger elements of the population in the first four as compared with the subsequent weeks of the epidemic. This appeared to be a reflection of the gradual extension of infection from children to adults. Particularly high attack-rates with explosive epidemics were seen in military establishments and residential schools. There also appeared to be a high incidence of the disease among nurses.

It was difficult to explain the precise reasons for the variation in the attack-rates or to discern details of the mode of spread of the virus. Dispersion appeared to limit, and close contact and crowding of persons together, particularly in dormitories or barracks, to favour the spread of infection.
2.3.1.4 The post-epidemic phase

In many countries the main epidemic phase was succeeded by a state of apparent quiescence of infection. Nevertheless, the Asian virus continued to be isolated month after month, or serological evidence of infection was obtained in sporadic cases. A definite recrudescence of clinical influenza occurred in the Netherlands in January-May 1958, two months after the end of the main epidemic. In the USA and also in Britain, a phase of excess mortality out of proportion to the morbidity from influenza occurred three or more months after the general epidemic. In Japan, in India and in certain areas of the USSR, definite epidemics of influenza with a high morbidity developed five or six months after the first epidemic; these were due to the same Asian virus. The geographical areas attacked in Japan and in the USSR during this second outbreak of infection were often those lightly involved in the first epidemic. There was evidence also that most persons who suffered from influenza in the first epidemic escaped during the second outbreak. The precise reasons for the varied experience of different countries during the post-epidemic phase could not be ascertained.

The Committee considers that the experiences of the 1957 pandemic of influenza show the need for further research. The epidemiological factors which determine the speed of spread of infection are at present obscure and can only be determined by further observations combined with laboratory studies. Until these factors are more clearly known it appears to be impossible to prevent the spread of infection, except by a limitation of human contacts.

2.3.2 Influenza in animals as a possible reservoir for human influenza virus

2.3.2.1 Animal viruses of the influenza and other groups (see also section 4, page 30)

Viruses belonging to the type A influenza group have been recovered from a number of species of animals in which they have caused epizootics.

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1 This subject was considered at a joint session with the Joint WHO/FAO Expert Committee on Zoonoses. For easy reference the relevant section of the report of that committee is reproduced below:

"Animal influenza and its possible relationship to human influenza"

"The Committee welcomed the opportunity of considering this subject in a joint meeting with the WHO Expert Committee on Respiratory Virus Diseases. The report of the latter deals extensively with the influenza problem in general, and the following section considers only briefly one component of the question.

"A clinical swine influenza infection has been known to exist in the swine population of the United States of America since the severe human pandemic of 1918. A virus was isolated as the causative agent in 1931 and was of the type A (common soluble antigen) of influenza viruses. Some influenza authorities believe that the infection in swine originated from human sources, but the possible role of swine or of other animals as inter-epidemic reservoirs of the human influenza virus has been the subject of much specula-
The swine influenza virus isolated by Shope in the USA is the best known of these agents since it was the first influenza virus isolated. There is epidemiological evidence that outbreaks of swine influenza occurred explosively in the Middle West of the USA for the first time in 1918. There is also serological evidence in man suggesting that this virus, or one closely related to it antigenically, was the cause of the pandemic of human influenza in 1918. With the exception of one unconfirmed report from Czechoslovakia, influenza viruses closely related to Shope’s original strains have been recovered from swine only within the USA. Viruses more nearly resembling human influenza viruses have been recovered from swine in Great Britain.

Epidemics of equine influenza in Czechoslovakia and elsewhere, during and after 1956, have yielded a virus possessing the soluble CF antigen of type A but having no other apparent antigenic relationship to human virus. Viruses belonging to the influenza A group have also been recovered from ducks in Czechoslovakia and Great Britain. The virus of classical fowl pest (fowl plague) also possesses the soluble A antigen. No epidemiological relationship has been found between the viruses of horse, duck and fowl influenza, and those of the disease in humans.

Among the viruses in the other groups, the Sendai virus (haemagglutinating virus of Japan) has been found in that country to be endemic in pigs, guinea-pigs, hamsters and mice. It is endemic in mice in China. A definite outbreak of influenza associated with a similar virus occurred in Vladivostock in 1956, and illnesses also occurred in children near Moscow.

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Consequently, when the human pandemic of 1957, caused by a distinctly new variant of type A (A2 = A/Aslan), began in the Far East early in the spring of 1957, WHO took the opportunity of organizing an animal serum survey in some 25 countries throughout the world. The immediate purpose of the survey was to determine if the A2 strain, or a closely related one, existed in the animal population before the pandemic struck a particular country; and, if not, whether the strain would establish itself in domestic animals known to be susceptible under natural conditions to other strains of type A virus. Furthermore, it was hoped to gain some preliminary information on the status of other type A strains known to exist in horses and swine in some countries.

"The results of this preliminary survey were still incomplete when the Committee met, but it was agreed that the information already gained certainly warranted continuation and expansion of analogous surveys, and specific research to elucidate the possible role of animals in human influenza. It is now known, for example, that distinct disease entities caused by type A influenza strains occur in epizootic form in horses (caused by A-equine/Prague/56 virus) in central and northern Europe, with serological evidence of its presence in the USA, where a type A infection in swine is well known. The virus of classical fowl pest (fowl plague) and two strains of viruses isolated from ducks all belong to type A. (The Sendai virus (haemagglutinating virus of Japan) has been isolated from pigs, hamsters and mice in Japan, and from mice in China, and has been reported to cause human disease in the USSR. This virus was formerly included with influenza strains as type D, but is now considered to be separate from the influenza group.) The significance of these findings cannot be evaluated at the present time and the problem requires careful investigation. The Committee strongly recommends that both WHO and FAO continue to encourage and coordinate research in this field." (Wild Hlth Org. techn. Rep. Ser., 1959, 169, 47)
later in the same year. Some of the latter illnesses were fatal. The strains of Sendai virus from the USSR were not at first mouse-virulent and required several passages before they produced lung lesions. The importance of the Sendai virus to human illness is, however, obscure. Serological evidence suggesting widespread occurrences of Sendai infection in man is obscured by cross-antigenic relationships with mumps and the viruses described in the USA and named croup-associated (CA) and haemadsorption viruses. Haemadsorption virus type 1 has been recovered from human beings and cattle in the USA and may be etiologically involved in the cattle disease complex known as shipping fever.

2.3.2.2 *Relationship between Asian influenza virus and infection in animals*

There has been speculation concerning a possible origin of the Asian influenza virus from an animal reservoir, both because of its unusual antigen and because of its insensitivity to serum β inhibitor. WHO has already initiated an investigation into this problem. However, no evidence has been received that any epizootic occurred during or before the emergence of the Asian virus in China. It is reported that virus of the Asian type was recovered from pigs in Manchuria in June 1957, and also that serological evidence suggestive of Asian virus infection was obtained after an outbreak of illness in horses and their attendants in Kharkov during October 1957. The animal attendants developed influenza during the Asian influenza epidemic in Kharkov, and after an interval of five or six days symptoms were noted among the horses. It is reported that the latter had high titres of HI antibodies to the Asian virus, and complement-fixing antibodies to the soluble antigen, some weeks after the outbreak, and that such antibodies were not found in sera from horses in other parts of Kharkov. Evidence exists that Asian virus antibodies were also demonstrable in sera collected from some horses in Moscow late in 1957 but not in those collected at an earlier date. No other unusual epizootics were reported from other countries during the Asian virus epidemic in man.

Deliberate attempts have been made by some workers to infect pigs with the Asian influenza virus, and a condition resembling the mild filtrate disease produced by the swine virus has been elicited. It is reported that virus resembling the Asian strain was recovered from the lungs of pigs in Spain after the human epidemic, but such sporadic isolations of human influenza viruses have been reported before. Serological surveys with sera from pigs have been made on a small scale in several countries, with results which fail to suggest that any widespread infection by the Asian virus has occurred in these animals.

There is a need for further investigations of a serological and of an experimental character, similar to those already organized by WHO, in order to establish the exact relationship between human and animal
influenza viruses, and the Committee strongly recommends that such studies should be made as soon as possible. Much work is needed before any statement concerning the possible existence of an animal reservoir for human influenza virus can be made.

2.4 Pathology

2.4.1 Influenza pneumonia

The Committee urges the need for further careful histopathological investigation of influenza virus pneumonia in the human being and in experimental animals. During the 1957 pandemic, in many countries fatal cases of influenza pneumonia were observed in which there was no apparent coincident bacterial infection. Microscopical examination of lungs from such virologically confirmed cases has shown lesions resembling in detail those seen in fatal cases during the 1918 pandemic of influenza and in sporadic cases in the intervening period. Though influenza virus pneumonia occurred sporadically in previously healthy children and adults, it was generally found, in 1957, that the majority of these virus pneumonias occurred in patients with pre-existent chronic pulmonary congestion as a consequence of chronic heart disease, lung disease or late pregnancy. The explanation of this experience is unknown.

Since mortality from influenza pneumonia during the recent pandemic was generally low, the Committee tentatively concludes that the 1957 Asian virus, though it had a potential affinity for the human lung tissue, did not exhibit a high level of virulence. The Committee suggests that in future influenza epidemics pathologists throughout the world should collaborate with virologists in order to detect and study cases of primary influenza virus pneumonitis. Such cases should be promptly reported to the national influenza centre.

The Committee also suggests that the properties of the infecting strains should be carefully watched in the course of future influenza epidemics, and that potential pneumotropism and pneumotropic virulence of isolated strains should be studied experimentally during the course of an epidemic. Study of the sensitivity of the virus to inhibitors present in normal human and animal sera is also encouraged in order to attempt to correlate such behaviour with the pneumotropic properties of the strain.

2.4.2 Secondary bacterial infection in influenza

Secondary bacterial infections have been reported to occur rather frequently in fatal cases of Asian influenza, and the various reports have shown a striking uniformity in the bacterial species involved in secondary infections. Death from secondary invasion by Haemophilus influenzae was rare. Pneumococcal and staphylococcal pneumonias were most
frequently encountered. Both in England and in the Netherlands *Staphy-
lococcus aureus* was cultivated from about 60% of fatal cases. In the
Netherlands this organism was found in many cases with microscopical
evidence of influenza virus lesions. In the USA and the USSR the incidence
of staphylococcal pneumonia seemed somewhat less.

In the Netherlands and the United Kingdom, 60%-70% of the infecting
strains of *S. aureus* recovered early in the disease were sensitive to penicillin,
while when recovered from hospital cases during the late stages of the
disease, only 40%-50% of the strains were so sensitive. This suggested
a hospital source for the infecting organism in a proportion of the cases.
In addition, hospital outpatients showed a greater occurrence of penicillin-
resistant strains than did patients who had no contact with a hospital
environment.

Certain epidemiological investigations, aided by phage typing, showed
that a staphylococcal pneumonia occurred frequently in influenza patients
who had a manifest staphylococcal skin lesion or who had contact with
family members or friends suffering from such lesions. Further studies
relating to staphylococcal complications and the means for prophylaxis
are recommended.

2.4.3 *Influenzal encephalitis*

In reports received from various countries, cases of encephalitis were
described as having occurred during the 1957 pandemic. These cases
were of particular interest because of the existence of certain laboratory
strains of influenza A viruses neurotropic for mice. There were a few
reports of isolation of strains of the Asian virus from the brains or cerebro-
spinal fluid of clinical cases of encephalitis, but difficulties exist in accepting
recovery of virus from the brain as a proved phenomenon unless strict
precautions have been taken at autopsy to prevent contamination with
material from the respiratory tract.

Considering the high morbidity of influenza during the pandemic and
the possibilities of coincident infection with other agents of neurological
disease, the reports of human cases with neurological symptoms or signs
were not considered to be excessive. The evidence did not therefore suggest
that the Asian virus exhibited definite neurotropic behaviour in man.

2.5 *Prophylaxis*

2.5.1 *Influenza virus vaccines*

Experience in many countries has now established vaccination as the
most efficient method for the prevention of influenza. A reduction in the
incidence of the disease of two-thirds or more has repeatedly been ob-
erved.
2.5.1.1 *Inactivated virus vaccine*

(a) *Choice of strains*

Agreement is unanimous that vaccines should contain a representative strain of the A2 sub-group or family of viruses currently prevalent. Some workers believe that for a time it is wise to include a strain of the A1 family because a co-prevalence of A2 and A1 strains may occur. Others prefer to broaden further the antibody basis of immunity by incorporating A and swine strains in a composite vaccine. For simultaneous protection against influenza B, a strain isolated since 1953 should be included. Again, some authorities recommend that vaccines should also contain the Lee strain of 1940. Vaccination against influenza C is not recommended at present.

(b) *Dose and route of inoculation*

For practical purposes a single one-ml dose of vaccine, given by the subcutaneous route, is advocated. The dose of vaccine used for immunization of children should be reduced in relation to age and body-weight. At all ages care should be taken not to inject the vaccine into egg-sensitive individuals. It has been suggested that in unusual circumstances, when vaccine is in short supply and the risk of disease is high, two 0.1-ml doses given intradermally at an interval of about two weeks might be tried. However, there is no evidence from field trials as to how effective such a procedure would be.

(c) *Indications*

From the clinical point of view protection is especially recommended in pregnancy, and for adults or children suffering from cardiovascular-renal disease, pulmonary disease and diabetes (see also section 2.4, page 14).

From the standpoint of public health practice, preference should be given to those persons concerned with the health services, public safety, transportation and essential trades (see also section 2.7, page 18).

(d) *Time of immunization*

Vaccination should, where possible, be carried out at least two weeks before the period when influenza is expected to be epidemic.

(e) *Type of vaccines*

To date, only aqueous vaccines have been adopted for mass immunization. Research on mineral-oil adjuvant vaccines continues to show promise. The Committee recommends that further investigation on the use of adjuvants of a variety of types be undertaken.
(f) Potency

The Committee recognized the limitations of in vitro tests for assaying vaccine potency. Haemagglutination techniques such as the chick-cell agglutinating (CCA) method are at times unsatisfactory, especially when applied to recent isolates. New methods for estimating the mass of antigen present in a vaccine should be sought. Meanwhile it is recommended that inactivated vaccines contain not less than 200 CCA units of each strain incorporated in the vaccine. It is also recommended that a comparative study of equivalence of the different units used in various countries be carried out.

2.5.1.2 Live virus vaccines

During the last decade there has been wide experience in the USSR with attenuated live influenza virus vaccines. The responsible investigators report effectiveness comparable to that obtained with inactivated vaccines. The principles guiding the development of live virus vaccines are: to produce a preparation that is maximally effective, is economic and can be administered easily, with the least discomfort to the recipient.

Recently isolated strains of types A and B influenza virus are attenuated by passage in embryonated eggs and may require passage in human volunteers or human lung-tissue culture to maintain infectivity. Acceptable strains must multiply in the respiratory tract of human beings and produce antibody in approximately 80% of vaccinated persons. Further, such strains must not produce febrile reactions in more than 0.5%-1% of subjects. Seed virus is then prepared and only one additional egg passage is allowed. For the finished vaccine, infected allantoic fluid is lyophilized and must retain a titre of $10^4$-$10^6$ 50% egg-infective doses (EID$_{50}$). Vaccine is reconstituted with appropriate diluent (saline or water). In general, the standard dose of one ml of reconstituted vaccine contains the virus present in 0.1 ml of allantoic fluid, although greater or lesser amounts may be recommended for use with certain strains. Desiccated vaccines have also been administered in powdered form mixed with suitable carriers. Vaccines are given intranasally by spraying or insufflation. When indicated, monovalent A vaccine is followed by monovalent B vaccine at an interval of not less than two weeks. Simultaneous administration of two strains is not recommended. Protection is believed to extend for six months after vaccination. Vaccination with these materials is not recommended for children of less than seven years old because the occurrence of severe febrile reactions is too frequent.

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1 As defined in: United States National Institutes of Health (1947) Minimum requirements: influenza vaccine, Bethesda, Md.
2.5.2 Serum prophylaxis

Investigators in the USSR have also reported protection, using hyper-immune horse serum prepared against recent isolates. Acceptable lots of serum at a dilution of one in ten must be capable of neutralizing at least 10,000 EID₅₀ of homologous virus in embryonated eggs. Serum has been employed in liquid or powdered form. The dose is one ml, or its equivalent, introduced intranasally at one- to three-day intervals during the period of exposure.

To date, experience with live virus vaccines and serum prophylaxis is largely limited to the observations of investigators in the USSR. Further studies are recommended.

2.6 Treatment

Since the publication of the report of the Expert Committee on Influenza in 1953, there have been few therapeutic advances of importance. There is at present no specific therapy for influenza or the other respiratory virus diseases considered in the present report.² Antibiotics have unquestionably proved life-saving in cases of influenzal pneumonia caused by bacterial complications, but their indiscriminate use in uncomplicated cases of influenza is hazardous. The Committee therefore wishes to emphasize the dangers of antibiotic-resistance which can result from such injudicious therapy, and stresses that there is no justification for the administration of antibiotics in uncomplicated cases of influenza in previously healthy persons.

Some authorities recommend the use of antibiotics even before chest complications have developed in patients suffering from various diseases and who contract influenza. Examples are patients with chronic pulmonary and cardiac disease. In other conditions, such as acute staphylococcal infections, diabetes, neurological disorders with respiratory involvement; and in late pregnancy, antibiotic therapy for cases of uncomplicated influenza requires discrimination and attention to individual circumstances. The treatment of influenza with secondary bacterial complications should be based on the causative organism and its antibiotic-sensitivity. Appropriate therapy therefore depends upon laboratory control. The scope of other therapeutic preparations used in certain countries requires further investigation; the preparations advocated include corticosteroids, hyper-immune horse serum, and iodine.

2.7 Public health measures

The Committee reviewed the recommendations regarding control measures against severe epidemics of influenza contained in the report

of the Expert Committee on Influenza in the light of experience gained during the 1957 pandemic. It was agreed that no substantial changes were indicated and that most of the opinions expressed had been justified in practice.

Quarantine measures were applied in several countries during the pandemic and were generally found to be ineffective, resulting at best in a short delay in the onset of the epidemic. The most striking example of the possibilities of quarantine was Israel, where the restriction or complete absence of international travel between that country and neighbouring States (due, not to deliberate quarantine restrictions, but to the political situation) seems to have resulted in a delay of about two months in the onset of the epidemic. In South Africa it was believed that some delay resulted from restrictions applied to ships arriving at ports, but this evidence was less convincing. Elsewhere no effect was detected. It seems that if such measures are to be effective, they must be very severe—so severe as seriously to interfere with international travel and traffic. This would be a high price to pay for a few additional weeks’ freedom from the disease, since there is no evidence that introduction can be entirely prevented. Such action could only be justified on technical grounds if the extra time permitted the application of effective specific prophylactic measures. It is, however, recognized that, confronted by a grave epidemic, health authorities might be forced by public opinion to take such action, even though it was likely to be ineffective.

The rather striking tendency in many countries for the 1957 pandemic to break out first in camps, army units, schools and other communities where contact between individuals was particularly close, as well as the reduced incidence observed in rural areas, suggests that the avoidance of crowding may be important in reducing the peak incidence of an epidemic, spreading it over many, rather than a few, weeks. This may be helpful in ensuring the maintenance of medical as well as other essential services. In the Northern Hemisphere at least, the opening of the schools after the summer holidays seems to have played an important role in initiating the main epidemic phase. In the face of a threat of a severe epidemic, closure of schools might therefore be considered. Such action is not recommended, however, in the type of influenza epidemic commonly experienced during the past decade.

As pointed out in section 2.5 (page 15), vaccination against influenza is now known to be effective in reducing the incidence of the disease. However in the event of an epidemic caused by a new virus, as in the 1957 pandemic, the value of this measure is limited by the speed of production of vaccine. Sufficient vaccine may be prepared in time to protect selected

\footnote{For easy reference the relevant part of the report is reproduced in Annex 1 (see page 34).}
groups if manufacturing facilities are available within the country. Experience in 1957 showed that practically no country was in a position to prepare in time significant quantities of vaccine for export after satisfying its own essential needs. Health authorities must therefore recognize that when such a pandemic recurs in the future, perhaps with a more severe form of the disease, they would have to rely largely on their own efforts for vaccine production. It is recommended that WHO should study this problem. The situation may be modified by the recent development of a live attenuated virus vaccine (see section 2.5.1.2, page 17) but further studies are needed before definite recommendations can be made.

The application of vaccination as a public health measure for selected groups is now a practical proposition. In general, vaccines prepared from strains of one family of viruses give reasonable protection against other members of the same sub-group. Major antigenic shifts of the influenza virus, while unpredictable, seem to have occurred at intervals of 10-15 years in the past. It is therefore probable that for several consecutive years at least, vaccines can be made which will give significant protection against the strains causing epidemics in the next influenza season. The problem is for whom, and how extensively, vaccination should be recommended against the current relatively mild influenza. Firstly, it may be strongly recommended that persons suffering from certain other diseases, listed in section 2.5 (page 16), should be immunized. Secondly, it seems advisable that nurses, physicians and hospital staff should be immunized. Thirdly, immunization may be recommended for personnel in public-safety and -utility services and essential trades and industries, as well as in special industries where a break in continuity of production results in severe economic loss. The Committee considered that advice on this latter application was beyond its terms of reference, and recommended that WHO should consult with the appropriate organizations, such as the International Labour Office, in order to arrive at more specific recommendations. Routine vaccination for the general population would not at present give results commensurate with the cost. However, use of vaccine on as wide a scale as possible is advocated should a virulent form of the disease appear.

During the 1957 pandemic it became clear that a major problem was the provision of adequate medical and hospital services. In many countries emergency medical services organized on an ad hoc basis seem to have worked reasonably well, but often were in danger of breaking down through illness, particularly among nurses. The personnel of such services should have a high priority for vaccination. Another problem became evident partly as the result of the excessive work-load on medical practitioners. Relatively mild cases of influenza which would normally have been treated at home were sent to hospital, where there is the danger of contracting fatal staphylococcal pneumonias caused by strains resistant
to antibiotics. Uncomplicated influenza cases should not be sent to hospital if adequate care can be organized in the home. On the other hand, it is also important to ensure that pneumonic complications are detected early so that treatment may be initiated in time. These two conflicting requirements are difficult to meet, but they must be borne in mind when organizing medical services for such an emergency as occurred in 1957.

2.8 The WHO Influenza Programme

The Committee reviewed the working of this programme since the first report of the Expert Committee. It was considered that the co-operation between the laboratories in many different countries participating in the programme and the World Influenza Centre in London and the International Influenza Center for the Americas had contributed much to knowledge of the epidemiology and control of influenza. However, it was pointed out that on a number of occasions the reporting of epidemics to WHO and the dispatch of virus strains to the international centres have been delayed or neglected altogether, even when there were signs that an epidemic or unusual strain of virus had appeared. Furthermore, there were still many countries and territories in which there were laboratories able to carry out the relatively simple procedures required by the programme, but which had not yet designated such a laboratory for official responsibility to co-operate in what must be, in order to be truly effective, a world-wide programme.

The importance of this is best shown by the fact that the recent pandemic began in an area not at present covered by the WHO programme. As a result, news of its onset did not reach WHO until more than two months later, after it had already begun to spread elsewhere.

It is pointed out that once news of the presence of this unusual epidemic (as it appeared to be at that time) was received by the United States Army and the WHO Influenza Centre Laboratories, the virus was isolated, sent to the appropriate reference centre and identified as an entirely new strain within three weeks, the information being broadcast at once to the rest of the world. This was a notable achievement. However, if the information had been received when it was already clear that a new and unusual virus was causing a severe epidemic, the rest of the world would have had two more months in which to prepare.

The Committee therefore recommends that WHO approach all countries with a view to seeking their co-operation in this programme. In doing so, it calls attention to the opinions expressed elsewhere in this report to the effect that, while it is impossible to predict the origin, extent or severity of influenza epidemics, certain public health measures which may have some effect in limiting the last two aspects may be possible if warning is received in time.
This programme, started by WHO in 1947 primarily as a research project, to learn more about the epidemiology of influenza, should now be regarded as an essential part of the world-wide public health defence against influenza. It is necessary, therefore, that the laboratory network originally organized under the programme should be brought into closer relationship with national public health authorities. This is necessary for two reasons—first, in order that the influenza centre of the country may be alerted to, and may organize the investigation of, outbreaks in distant parts of the country, of which it might otherwise not learn in time, and secondly so that the centre may keep the health authorities informed of the appearance of unusual viruses or epidemics elsewhere in the world and of the appropriate technical measures which should be taken. Under the existing arrangements the appearance of unusual viruses is notified to influenza centres and not directly to health authorities, since this is technical information which may require interpretation. It is recommended that this procedure be continued, provided that a clear working relationship can be established between the centre and the health authorities.

2.8.1 Epidemiological information

The Committee recognized that in the great majority of countries notification of clinical influenza either was not obligatory or was generally incomplete. However, the most important thing was not the enumeration of cases but detection as soon as possible of an unusual incidence of epidemic respiratory disease, which could then be investigated by the influenza centre in order to determine its nature.

The methods adopted to detect such outbreaks will vary from country to country. Some guidance on ways of detecting outbreaks or an unusual incidence of disease was provided in the report of the Expert Committee on Influenza.1

The importance of maintaining observation of schoolchildren was evident as a result of experience during the 1957 pandemic.

2.8.2 Extension of the WHO Influenza Programme to other respiratory diseases

As indicated on page 5, among the respiratory virus diseases of known etiology given primary consideration in this report, the only ones other than influenza that can readily be studied at the present time are infections caused by adenoviruses. It is recommended, therefore, that wherever possible influenza centres should undertake the diagnosis of such infections. It is suggested that WHO should consider the designation of international

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1 For easy reference the relevant part of the report is reproduced in Annex 2 (see page 35).
centres which might serve in the same way as the World Influenza Centre for the study of these infections.

In view of the fact that there are many other minor respiratory infections which are nevertheless of considerable socio-economic importance, and that there is an increasing number of newly discovered viruses apparently responsible for respiratory disease but of which the public health importance is not yet known, WHO should consider designating an international centre to assist in co-ordinated research.

On the grounds of efficiency and economy it is recommended that, as far as possible, such extensions of the WHO Influenza Programme should be integrated with the existing programme. As pointed out earlier, classification of respiratory disease by clinical differentiation alone is severely restricted, and it is only from combined clinical, laboratory, and epidemiological studies that further progress can be expected.

2.9 Developments in laboratory techniques

The laboratory techniques described in the report of the Expert Committee on Influenza ¹ have remained useful but require a few changes in detail. However, attention is drawn to difficulties which became apparent in the performance of the haemagglutination-inhibition (HI) test and to promising new methods for the diagnosis of influenza which, although not as yet extensively tried, may ultimately become useful additions to the techniques of the diagnostic laboratory.

For the isolation of influenza virus the chick embryo remains the preferred host. It has been reported that primary cultures of monkey renal cells are more sensitive to influenza virus on first isolation than is the chick embryo, that the presence of virus may be detected as early as 24 hours after inoculation by the fact that infected cells readily adsorb guinea-pig or other red cells,² and that this haemadsorption can be specifically prevented by strain-specific immune sera which may thus serve to identify the isolated virus. The greater sensitivity of monkey renal cells to influenza viruses may hold for certain strains, but has not been observed with A2 (Asian) virus. Furthermore, the haemadsorption phenomenon is also exhibited by certain simian viruses frequently found in monkey renal cultures, and this severely limits the usefulness of the technique for the isolation and identification of influenza viruses.

For the identification of new isolates of influenza virus and the determination of antibody levels in acute and convalescent, or pre- and post-vaccination, sera of man or animals, the HI test has remained the most

¹ For easy reference the description of these techniques is reproduced in Annex 3 (see page 36).
² Vogel, J. & Shelekov, A. (1957) Science, 126, 358
commonly used technique. While the HI test is convenient and seemingly simple, it is beset with many difficulties which have been particularly troublesome with the A2 virus. It is necessary to be aware of these difficulties in order to minimize them wherever possible.

The problems referred to concern the viruses as well as the sera employed in the HI test. Freshly isolated strains of virus often undergo changes on adaptation to laboratory hosts. In early chick-embryo passages the type A virus may be in the original (O) phase, i.e., it agglutinates human or guinea-pig red cells to considerably higher titres than chicken erythrocytes. After several passages it changes to the derived (D) phase, in which chicken red cells are agglutinated to the same or higher titres than human or guinea-pig cells. Other strains may lack affinity for antibodies (Q phase), in contrast to the avid strains (P phase). Furthermore most influenza strains are susceptible to one or more non-specific inhibitors of haemagglutination present to a varying extent in the sera of many animals and man. At least two such inhibitors are known which affect various strains of virus to different degrees. The non-specific inhibitors may be inactivated or removed from sera by certain procedures. The following methods have been employed, either alone or in various combinations: heating to 56°C, treatment with crude cholera filtrate, potassium periodate (KIO₃), trypsin or carbon dioxide. Of these techniques the use of trypsin has often been found to be disappointing, while that of periodate is most widely applicable. It should be noted that none of these methods, when used alone, affects all inhibitors alike. Furthermore, there is no assurance that the treatments do not also reduce specific antibody levels, particularly when the serum under test is of low titre.

These various problems have been in evidence again to a greater or lesser extent with strains of A2 virus. A2 strains isolated in chick embryos were almost all in the D phase but they have shown variability with respect to their affinity to antibodies (P-Q variation), and susceptibility to non-specific inhibitors of haemagglutination. It thus has been difficult at times to identify new isolates and to obtain satisfactory reagents (virus and sera) for the serodiagnosis by the HI test. While in the hands of experts these difficulties have been overcome in one way or other, the techniques employed in various laboratories differ considerably. In the absence of comparative tests employing the various modifications of technique it is not possible at this stage to suggest a method for general use. However, a few points should be emphasized. For the serodiagnostic HI test it is essential to use heated sera and to employ strains of virus which are antibody-sensitive (P phase) and relatively insensitive to inhibitors. Whatever methods are adopted in individual laboratories, it is strongly recommended that the sensitivity of the HI technique should be checked against the diagnostic

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1 Gorbunova, A. S. et al. (1956) Vop. Virusol., 2, 21
results obtained with another test, preferably a complement-fixation reaction (see below).

In view of the difficulties encountered with the HI technique, attention is drawn to recent reports concerning a strain-specific complement-fixation test, which was found to be particularly useful in the identification of new isolates of virus but may be employed also in the strain-specific serodiagnosis of the disease. It has long been known that there exist at least two complement-fixing antigens of influenza virus—the soluble (S) antigen and the virus (V) antigen. The S antigen is type-specific and identical for all strains of one type; the V antigens are strain-specifically oriented, and most probably identical with the haemagglutination components. It is self-evident that for identification of strains and for strain-specific serodiagnosis, V antigens must be obtained which are free of detectable S, and anti-V sera must be prepared which are devoid of anti-S. Such reagents can now be produced by relatively simple methods (see Annex 4, page 50). These reagents have been used in the various problems for which the HI test is at present employed without experiencing difficulties related to phase variation of the virus or non-specific inhibitors. It is now desirable to confirm these results and to test the technique on a larger scale before any recommendations can be made.

For routine diagnostic purposes, allantoic fluid which contains V and S antigens, or crude extracts of allantoic membranes which contain only S antigen, have given good results in many laboratories (see Annex 4, page 50, for a discussion of the advantages and disadvantages of this method).

With respect to the soluble antigen employed for type-specific diagnosis, it might be desirable to adsorb the preparation with chicken red cells. The S antigen is not adsorbed but the V antigen present in varying concentration is removed and thus interpretation of the results of the complement-fixation test will be uncomplicated by possible interaction of the V component with the corresponding V antibodies that also might be present in some of the sera used.

For the standardization of S antigen, a box titration is recommended employing falling dilutions of human or ferret convalescent sera against decreasing dilutions of S antigens. This is desirable because of the fact that prozones occur in this reaction in the region of antibody excess. By this means the optimal unit of antigen is determined, that is, the amount of antigen giving the highest serum titre. Two optimal units should be employed in the test.

For diagnostic purposes, neutralization tests in eggs or tissue cultures may also be used.

Among suggestions for additions and changes in procedures outlined in the earlier report the following deserve mention. It has been found in some laboratories that immune sera derived from rats lend themselves to identification of strains because the inhibitor in fresh sera is readily
removed by treatment with CO\textsubscript{2}. On storage at refrigeration temperature (4°C) sera may acquire inhibitory activity which is no longer affected by CO\textsubscript{2}. This experience also serves to re-emphasize the need for storage of sera either in the dried state or frozen at —20°C or lower temperatures.

In the preparation of cholera filtrate it was noted that two or three cycles of freezing and thawing of the cultures before filtration increases the yield of the enzyme.

3. ADENOVIRUS INFECTIONS

3.1 Etiology

The adenoviruses are a newly described family of viral agents, some of which are an important cause of acute respiratory, ocular and enteric infections of man and certain of the higher primates. There are at least 18 distinct antigenic types of adenovirus as measured by the serum neutralization technique. All the viruses of the family, however, share a common group-specific "soluble" antigen which is readily detectable by the complement-fixation technique.

3.2 Clinical Syndromes

The adenoviruses attack the respiratory tract in man from the nose to the lungs, the conjunctivae, and the intestinal tract. The lesions induced include cellular damage with inflammation and hypertrophy of the corresponding lymphatic tissue. The clinical picture seen in the individual patient is related to the particular tissues involved and to the degree of damage.

Most adult patients with adenovirus-caused respiratory disease present a basic syndrome of fever with pharyngitis and cough which is sometimes severe. This may be accompanied by conjunctivitis, coryza, exudate on the hypertrophic lymphatic tissue of the throat, lymphadenopathy, and lower respiratory-tract involvement including tracheobronchitis, bronchiolitis and atypical pneumonia. Constitutional symptoms such as headache, malaise and myalgia are commonly present.

The respiratory illnesses caused by the adenoviruses fall into the general category of the febrile catarrhs. More specifically, however, these agents have been shown to be etiologically related to the previously described syndromes of undifferentiated acute respiratory disease, non-bacterial exudative pharyngitis, atypical pneumonia and pharyngo-conjunctival fever. In addition to respiratory illness, certain of these agents may cause follicular conjunctivitis and epidemic kerato-conjunctivitis. Gastro-intestinal symptoms of a transient nature have been observed in some of the cases in certain outbreaks, especially in children.
3.3 Epidemiology

Adenoviruses have been recovered from human beings throughout the world. Although such agents have been isolated from animals, no important animal reservoir of the types found in human infections has been demonstrated to date.

Occurrence of epidemics of acute respiratory illness caused by these agents appears to be favoured by close contact and other conditions, such as are experienced by military recruits. The attack rates of febrile cases of the disease may reach 25% during the winter season among new recruits, but the rates in the summer are usually low. Among the civilian population the disease is definitely less common, but summer epidemics of types 3 and 7 infections have been observed, especially among children. The latter epidemics may have been related to exposure to contaminated water in swimming-pools or lakes.

It may be pointed out, in this connexion, that the adenoviruses may be spread not only through the air but also by mechanisms common for the enteric viruses. Thus, the virus may be excreted in stools in large quantities for long periods of time.

The various types of adenovirus may show a marked predilection for certain sections of the population. Among infants, types 1, 2, 3, 5, 6 and 7 are encountered most frequently. Among older children, types 3 and 7 virus have been found most often. Among recruits in military camps in the USA and the United Kingdom, types 4 and 7 have been most important, while type 3 is sometimes found. Type 14 has, to date, been epidemic only in the Armed Forces of the Netherlands. Among adult civilians, types 3, 4 and 7 are the most frequent, although other types have been found. Type 8 has been causally related to epidemic keratoconjunctivitis.

3.4 Laboratory diagnosis

The same basic principles used for laboratory diagnosis of other viral infections apply to the diagnosis of respiratory illnesses caused by adenoviruses. Specifically these are: (1) recovery and identification of the causal agent, and (2) demonstration of an increase in antibody titre between acute and convalescent sera.

3.4.1 Virus recovery

The adenoviruses have been propagated, to date, only in tissue cultures of human or of certain animal cells. For isolation purposes, (a) primary explant cultures of human amnion or human respiratory tissues or (b) serially propagated cell cultures usually of malignant origin (e.g., HeLa, KB), have been used successfully. Cultures of animal cells have generally proved incapable of propagating adenoviruses of human origin in series.
However, adaptation of certain adenovirus types to monkey-kidney cell culture has been accomplished with difficulty.

To recover virus, throat washings collected from the patient during the first three days after onset of illness are inoculated into tissue cultures. The virus may also be excreted in the stools, even for long periods following recovery from the disease. Presence of virus is indicated by the appearance microscopically of a typical cytopathic change in the cells. Intranuclear inclusions may be observed in suitable stained cultures. With inocula containing only a small quantity of virus, the incubation period before the appearance of cytopathic change may be long. This is related to the long growth-cycle, characteristic of these agents. For virus recovery, the cultures may be held for long periods, up to one month, or, alternatively, blind passage may be made at from three- to four-day intervals for up to five passages. By either method, successful recovery may be accomplished from the respiratory secretions from up to 75% of serologically proved cases of the disease.

Recovered viruses may be identified as belonging to the adenovirus group by the complement-fixation test, or the specific virus type may be determined in neutralization tests performed in tissue culture. In practice, it is well to establish, first, the group identity of the isolate by tests for the group “soluble” antigen in the culture employing the complement-fixation technique and using human convalescent serum as the source of antibody. For typing strains, monotypic antisera prepared in rabbits by repeated intravenous inoculation of live virus are customarily employed.

3.4.2 Serology

Serological diagnosis of respiratory disease caused by adenovirus may be made by either the complement-fixation or the neutralization technique. Where a group diagnosis suffices, the simple in vitro complement fixation method is preferred. Where indication of the infecting type is required, the neutralization technique can be used. This test is far more costly and laborious than the complement-fixation procedure, requiring an available supply of tissue cultures and virus seed stocks representing the various types. In most instances, types 1-7 plus 14 will suffice for diagnosis of adenovirus-caused respiratory illness. Although the neutralizing antibody response in the patient is usually greatest against the homologous type, this is not always the case. Hence, the test has less validity in establishing the infecting type than has the recovery and identification of the agent itself.

For serodiagnosis, the first blood sample is withdrawn as early as possible after onset of illness, preferably within the first three days. The convalescent specimen is withdrawn at two to three weeks after onset. Neutralizing antibody usually achieves the maximum level by three weeks,
while the greatest titre of complement-fixing antibody may be reached by the end of the second week.

The complement-fixation test may be performed by any of the usual procedures, provided that the reagents are properly standardized (see section 2.9, page 23, on influenza diagnosis). Certain laboratories prefer fixation at 36°C for one hour, while other laboratories incubate the tests overnight in the cold (4°C). Fixation at 36°C is less sensitive, giving lower titres, but is more rapid and may cause less non-specific fixation of complement. All titrations of paired sera must be carried out simultaneously in the same test and all tests must include a control antigen. In the tests, a fourfold or greater increase in amount of complement-fixing antibody in the second serum sample as compared with the first is considered significant.

The antigen for use in the complement-fixation test is prepared in tissue culture, most often in HeLa, KB or monkey-kidney cells. For maximum release of soluble antigen, the cells must be disrupted by three alternate freeze-thaw cycles or by trituration in a blender or homogenizer. Non-infected antigen for control purposes is prepared at the same time in the identical fashion. The materials are clarified by low-speed centrifugation (3000-5000 revolutions per minute (r.p.m.) for 20 minutes). The complement-fixing antigens usually titre between 1:8 and 1:32, and are stable for long periods when stored frozen at -20°C.

The antigens are generally employed as live virus material, although some workers may prefer to heat the preparations at 60°C for 30 minutes.

Serum-neutralization titrations of the paired sera are tested by a standard procedure employing a representative group of virus types. Special population groups have been attacked by certain viral types in particular, and attention to this matter may be of help in reducing the number of types which are to be employed in the tests (see section 3.3, page 27). Demonstration of a fourfold or greater increase in amount of neutralizing antibody is considered to be significant. Increase in titre may also be judged from prolongation of the time before cytopathic changes appear in the tissue culture.

3.4.3 Caution regarding interpretation of laboratory diagnostic test results

It must be stressed that in isolated cases of a disease, a positive diagnostic test can be regarded only as indicative of adenovirus infection during the time period of the collection of specimens from the patient and does not necessarily prove an etiological relationship to the illness. For proof of etiological significance in a particular disease, statistical correlation in a

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4 Rowe, W. P. et al. (1955) Amer. J. Hyg., 61, 197
controlled study must be obtained. Thus far, the etiological relationship
to respiratory disease has been definitively proved for only a few of the
adenovirus types, the best established being for types 3, 4 and 7. Type 8
has been causally related to epidemic kerato-conjunctivitis. Attention is
called to the danger of laboratory contamination of cultures with aden-
viruses where these agents are already under study in the laboratory.

3.5 Prophylaxis

Vaccines for prevention of acute respiratory illnesses caused by adeno-
viruses have been developed and evaluated in controlled field studies
conducted in epidemics in military recruit camps. Vaccines so far prepared
have been bivalent, containing virus types 4 and 7, or trivalent, containing
types 3, 4 and 7. The vaccines ¹ are prepared from infected monkey-kidney
cell tissue cultures kept in a serum-free maintenance medium. The viruses
are inactivated with formaldehyde.

Both vaccines induce significant neutralizing antibody against the
homologous agents after a single intramuscular injection of 1 ml. Bivalent
types 4 and 7 vaccine may induce considerable antibody against type 3
virus as well, and this has been shown to be due to the type 7 component,
which is related immunologically to type 3 virus.

Extensive field trials of both the bivalent and trivalent vaccines carried
out among military recruits proved their high level of effectiveness in
preventing febrile respiratory illness caused by type 4 or by type 7 adeno-
virus. Type 3 vaccine has not as yet been evaluated. In the studies the
vaccines effected an over-all reduction in total respiratory illness of the
order of from 55% to 81%. The reduction in cases caused by adenovirus
only was in the range of from 83% to greater than 90%.

Use of adenovirus vaccine is justified among new recruits in the armed
forces wherever there are high attack rates; among the civilian population
and the permanent military personnel, these have generally been so low
as to provide no real basis for general use of the vaccine.

4. MISCELLANEOUS RESPIRATORY VIRUSES

4.1 Myxoviruses — non-influenza ²

Knowledge about the viruses in this group and their relationship to
disease is at present fragmentary (see also section 1, page 3, for addi-

¹ Information concerning commercial manufacture of adenovirus vaccine has been
published in the Federal register of the United States General Services Administration

² The Virus Sub-committee of the International Nomenclature Committee is con-
sidering renaming the Sendai, CA, HA1 and HA2 viruses *myxovirus para-influenzae*
types 1, 2, 3 and 4 respectively. The use of the term influenza D for Sendai virus being
specifically discouraged.
tional information). The Sendai virus (haemagglutinating virus of Japan) has been reported to cause pneumonitis in children and influenza-like disease in adults. It can be recovered by intra-allsanto inoculation of embryonated eggs.

The haemadsorption (HA) viruses types 1 and 2 have been recovered in monkey-kidney tissue cultures and are so called because they can be detected in such cultures through adherence of red blood cells to the infected cells in the culture (haemadsorption) before cytopathic change is apparent. HA type 1 virus has been recovered from children with pharyngitis, bronchiolitis and pneumonitis. HA type 2 virus has been recovered from children with croup (acute laryngo-tracheo-bronchitis). These agents may also be associated with other minor respiratory illnesses in children. The HA viruses are antigenically related directly to each other, and type 2 virus is related to the Sendai virus. CA or croup-associated virus, recovered from children with croup, grows in monkey-kidney cell tissue cultures causing cytopathic effects, and agglutinates red blood cells. Antibodies against the agent are present in many human sera, and significant antibody increase against the agent occurs in some cases of croup in childhood.

4.2 Respiratory syncytial virus (CCA and Long strains)

This virus has been recovered from chimpanzees with coryzal illness and from infants with croup or pneumonia. The virus grows only in certain lines of human cells in tissue culture. Definitive evidence for causal relationship to human disease is wanting.

4.3 Atypical pneumonia virus (Eaton's agent)

This virus grows in bronchial epithelium of chick embryos and may be demonstrated by means of fluorescein-labelled antibody. Probable relationship to atypical pneumonia associated with the development of cold-agglutinins has been shown.

4.4 JH and 2060 viruses

These viruses are apparently similar antigenically, although not identical. They have been recovered from military recruits (2060) and from children (JH) with mild upper respiratory illness resembling the common cold. Attempts to produce colds in volunteers given the JH virus have yielded equivocal results. Both agents may be propagated in tissue cultures of monkey kidney.
4.5 Psittacosis and Q fever

These agents, which are important causes of atypical pneumonia in man, are dealt with more fully in the report of the Expert Committee on Zoonoses.

4.6 Common cold

Agents causing the common cold syndrome have been demonstrated definitively, as yet, only in experiments with human volunteers. Attempts to propagate cold virus in tissue cultures have produced equivocal results.

4.7 Enteroviruses

Coxsackie A virus causes herpangina, and Coxsackie B types have been associated with epidemic pleurodynia or Bornholm disease and certain other syndromes. Certain of the ECHO viruses have been found in association with human respiratory illnesses, for example, types 10, 11 and 20.

5. SUMMARY AND CONCLUSIONS

The Expert Committee which met in Geneva in 1952 dealt only with influenza. At its present meeting the Committee on Respiratory Virus Diseases has also considered other respiratory infections. However, the 1957 pandemic of Asian influenza was an event of such importance that a large part of the discussions of the Committee were devoted to the lessons of that pandemic and the results of the study of influenza in recent years.

5.1 Influenza

The origin of the pandemic was apparently in the Kwei-Chow province of China in February 1957. Influenza virus A is known to change somewhat in its antigenic and other properties from one outbreak to another; but the novel A2 or Asian virus represented a quite radical departure from the make-up of earlier strains. This was so great that few people had any antibodies against it and the new strain spread around the world within six months. Fortunately its virulence was low and nowhere was the mortality alarming. The report discusses the varying behaviour, particularly as regards speed of spread, in different countries, and has drawn attention to the correlation of climate, and degrees of crowding with morbidity. There has been discussion also of the origin of the epidemic, whether from an animal reservoir or otherwise, and of the possible relationship to the agent causing the 1889 influenza pandemic; this matter has arisen
because of the presence of antibodies to A2 virus in some persons who were alive in 1889 and their virtual absence or low titre in younger people.

The study of the behaviour of the epidemic and the tracing of its course have only been possible through the co-operation of workers all over the world, both within and without the network of WHO influenza laboratories; the WHO Influenza Programme has, in fact, been abundantly justified.

Vaccination studies have shown that much influenza can be prevented, even when vaccine against a new strain has to be made quickly.

In the USSR attention has been devoted mainly to elaborating an attenuated live-virus vaccine: in most other countries a formalin-inactivated-virus vaccine has been used. The reduction in incidence has, however, been generally of the order of two-thirds, never complete.

Much remains to be done. Coverage of the world by the WHO network should be made more complete, and its operation made speedier and more efficient. Diagnostic methods should be made simpler and more reliable. Vaccines need to be improved, and new or more effective means of treatment and control should be sought. Much can be done in the way of epidemiological studies to reveal the reasons for the varying behaviour of the disease. Serological study may yet reveal the secret of the origin of epidemics and the possible relationship of their causative viruses to similar animal viruses, and to those of earlier pandemics.

5.2 Other respiratory infections

Quite apart from influenza, upper respiratory infections are a major cause of morbidity in all countries. Most of them are of unknown etiology, though new respiratory viruses are being brought to light almost every month. It is of urgent importance to evaluate the significance of these in public health, and to know how to study and control those as yet undiscovered. Much has been learnt concerning adenoviruses and how to protect against them; but they seem to represent only a small part of the "nuisance viruses" which confront the world population. The common cold still represents the biggest unsolved problem; however, none of the newly revealed viruses can be reasonably incriminated as the cause of this baffling syndrome. A useful contribution by WHO may well be to help in arranging for the study, comparison and distribution of newly-isolated respiratory viruses.
Annex 1

CONTROL MEASURES AGAINST SEVERE EPIDEMICS
OF INFLUENZA *

1. General measures

The control measures which have been used in the past for combating particularly severe epidemics of influenza are based first of all on standard public health procedures. These include quarantine (see below), restriction of movement of individuals, avoidance of crowds in cinemas, public meetings, etc., and, of most importance, the provision of extended hospital services. Other measures, such as the wearing of masks, and adequate ventilation and disinfection of the air (aerosols and ultra-violet light) in selected buildings are of more doubtful value. Public anxiety in the country affected and in other countries must be allayed as far as is practicable. In regard to quarantine, the committee felt that the isolation of groups within a community has not been shown to limit the spread of infection. Quarantine at a national level would be likely to be effective only in the case of islands and geographically isolated communities. The role of quarantine in regard to large countries or continents cannot be assessed while knowledge concerning the spread of influenza is still imperfect. There is no evidence, however, that in the case of such large areas quarantine could be applied effectively. The probable value of restriction of travel between countries can be judged in the light of the above remarks; and, particularly in the case of mild epidemics of influenza, it is totally unjustified.

2. Role of vaccines

The committee is hopeful of the use of vaccines in relation to epidemics, but the limiting factor in the utilization of this measure is the speed of production. Pharmaceutical houses are reluctant to prepare for storage supplies on a mass scale which in any case might not be of the proper antigenic type. It is clear, however, that if there were a dangerous world-wide epidemic it would be impractical to manufacture enough vaccine from eggs for universal use. Key persons could, however, be immunized; and economy in the quantity of vaccine might be attained by intracutaneous inoculation or by the use of adjuvants. The possible use of a live non-attenuated virus vaccine, either alone or with simultaneous injection of antibody, could hardly be recommended in epidemics such as those currently experienced but might be considered practicable in the face of a

really lethal outbreak. In any case, virulent epidemics such as the 1918 pandemic particularly affected certain age-groups; and these might, therefore, be selected for attempted protection in such an event in the future. The committee recommends that WHO should carry out a survey of the potential production capacity of influenza vaccines at present available.

Annex 2

COLLECTION AND DISTRIBUTION OF EPIDEMIOLOGICAL INFORMATION REGARDING INFLUENZA *

It has already been pointed out that the virus of influenza shows no stability in nature, and that at present it is impossible to foresee when a serious epidemic may occur. If it should occur, the condition spreads with such rapidity that there is very little time in which to initiate control measures. The committee therefore stresses the need for the national administration to obtain early information of the occurrence of an outbreak of influenza within its territory, and within neighbouring territories and other countries with which it is in frequent contact, in order to give itself, and others, time for the institution of the practical measures which can be taken to meet the emergency created by an epidemic of the disease. Early knowledge of the strain of virus involved is also necessary if specific vaccines are to be used to protect selected groups in the community.

The committee agrees that the standard practice of notifying (reporting) individual cases, which is appropriate for diseases such as typhoid fever, is inadequate for influenza because many cases are not notified, since they escape medical attention or because practising physicians are too overworked to report each case. It is also pointed out that in many countries influenza is not a notifiable disease; in others, only epidemics are reported; in still others, individual cases must be notified. Therefore, figures purporting to record actual numbers of cases mean very little, and there can be no comparison between the figures originating in different countries. What is needed is a way in which an abnormal prevalence of influenza (or influenza-like conditions) can be detected promptly and the course of the epidemic followed with the minimum time-lag. This might be done in a given area by the preparation in advance of a rough scale—to indicate the incidence of the disease—based upon local information which could readily be obtained, such as the daily records of absenteeism in one

or more industrial plants, or among public transport workers; absenteeism in schools; the volume of new insurance claims where social insurance covers the bulk of the population; requests for admission to hospital (especially for pneumonia), etc. A scheme by which a selected sample of practitioners voluntarily report to the local health authority the number of influenza-like diseases treated daily has recently been introduced experimentally in some countries and may prove very valuable. It is, of course, a matter for national health authorities to collect such information without delay from strategically located towns or areas. Such information should be notified promptly (telegraphically in time of epidemics) to the epidemiological services of WHO, which would in turn make the information available to other health administrations through its daily epidemiological broadcast bulletins and the several epidemiological weeklies issued and airmailed from Geneva, Washington, Alexandria, and Singapore. The information would also be passed on by WHO to the WHO network of influenza laboratories. This is necessary to initiate measures to isolate and identify the responsible virus. The results of these efforts would be made available as soon as possible to all national health administrations through the epidemiological services of WHO.

Annex 3

SEEROLOGICAL AND DIAGNOSTIC TECHNIQUES

A. METHODS OF PREPARATION OF ANTISERA FOR THE COMPARISON AND TYPING OF STRAINS OF INFLUENZA VIRUS *

1. Preparation of ferret antiserum

After a preliminary removal, under anaesthesia, of blood from the heart, two ferrets are inoculated intranasally with 1 ml of a 10⁸ dilution of freshly harvested, infected, allantoic fluid. The animals are kept under strict isolation and are bled out 12 days after the original inoculation. To each serum after separation 1/10 000 Merthiolate is added, and the sera are then stored at 2°C. Alternatively the sera may be stored at —70°C or lyophilized.

After treatment with cholera filtrate, the pre-infection serum specimen is tested for influenza antibody in order to exclude natural infection of the ferret before inoculation. The post-infection serum can be used for strain analysis only if the pre-infection serum is devoid of specific inhibitory activity in the haemagglutination-inhibition test.

Treatment with cholera filtrate as prepared below (see page 38) is considered necessary by some workers. One volume of serum plus four volumes of crude cholera filtrate are incubated overnight at 37°C; the mixture is then heated for 1 hour at 56°C in order to destroy cholera enzyme activity. Other workers (Burnet & Stone 1) recommend treatment of ferret antiserum with the receptor-destroying enzyme of *Vibrio cholerae*, which is prepared from cholera filtrate by absorption with 5% fowl cells for 1 minute at 2°C followed by elution into normal saline for 30 minutes at 37°C. The eluate is diluted in acetate buffer, pH 6.0, and titrated for its ability to destroy non-specific inhibition in normal ferret serum using a technique similar to that described below. The eluate is then used with immune ferret serum at the highest effective dilution. This dilution is mixed with serum and the whole is incubated overnight at 37°C, followed by heating at 56°C for 1 hour.

2. Preparation of rabbit antiserum

The antigen consists of infected allantoic fluid from embryonated eggs. Ten-day-old eggs are inoculated into the amniotic sac with 0.25 ml of a 10⁻⁴ dilution of high-titre, egg-adapted seed virus. The inoculated eggs are incubated for three days, after which they are cooled and the allantoic fluids are removed and tested for haemagglutinating activity. Fluids of high haemagglutinating titre are pooled and tested for bacterial contamination (no antibiotic or other antibacterial material is employed).

Healthy young adult rabbits of either sex are inoculated intraperitoneally three times at weekly intervals with 5-ml quantities of whole, infected allantoic fluid, and are bled one week after the third inoculation.

The crude serum is treated for non-specific inhibitors in the manner described below.

3. Preparation of fowl antiserum

The antigen is derived from embryonated eggs, previously incubated for 9 days at 39°C, which are inoculated with 0.4-ml amounts of the appropriate dilution of seed virus (usually 10⁻⁴ infected allantoic fluid)

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into the allantoic cavity. After inoculation the eggs are incubated at 35°C for 40-44 hours, and the allantoic fluids are harvested after chilling in the refrigerator. The haemagglutination titre of the fluid for immunization should be at least 1/320 (initial dilution); titres of 1/640 or 1/1280 are desirable.

After preliminary bleeding, 5- to 8-pound (2- to 3½-kg) roosters (cockerels) are injected with 5 ml of the infected allantoic fluid intravenously and 10 ml intraperitoneally. For newly isolated strains in which the haemagglutinin titres may be low (1/320) it is best to repeat the injections on the following day.

Ten days after the initial injection, the chickens are bled from the heart. (With large roosters, 80 ml can be removed on 3 successive days.) The blood is allowed to clot, is cut into small pieces, and is allowed to stand overnight in the ice-box, followed by 5 or 6 hours at room temperature (to aid contraction of clot). The serum is removed, inactivated at 56°C for 35 minutes, and lyophilized in 0.5-ml or 1-ml amounts and stored at +4°C. Storage of the undried serum at −20°C may also be acceptable.

The pre- and post-immunization serum specimens are titrated against the homologous antigen in order to determine the agglutination-inhibition titre. The immune serum should have a titre of at least 1/800 (initial dilution) with homologous antigen; 1/400 may prove acceptable. The pre-vaccination serum should not inhibit in a dilution of 1/50. Most viruses are not inhibited by normal rooster serum diluted 1/50, but exceptionally a non-specific inhibition titre of 1/200 may be encountered with newly isolated strains. For strain analysis, sera must be treated to remove non-specific inhibitor by incubating for 6 hours at 37°C, with 4 volumes of crude cholera filtrate prepared as indicated below. The cholera enzyme activity is finally destroyed by heating at 56°C for 50 minutes, and there is a demonstrable loss of antibody.

Strain analyses are carried out by the standard haemagglutination-inhibition method, using human red cells. In strain analysis, it is essential that the tests be read after a standard incubation period such as 55 minutes.

4. Preparation of crude cholera filtrate for the destruction of inhibitors

Preparation of culture

The strain, V. cholerae 4Z (Burnet & Stone), is cultivated in nutrient agar containing 2% agar and 2% peptone, pH 7.6, subcultures being made every 3 weeks. Subcultures yielding a potent enzyme should be lyophilized as the potency in terms of enzyme production is inconstant and may be decreased by serial subculture.
The seed culture for the preparation of a filtrate is a 6- to 8-hour growth in nutrient broth containing 2% peptone at pH 6.9. From this culture agar plates containing 0.8% agar and 2% peptone (pH 6.9) are inoculated and incubated for 16 hours. A heavy growth should be obtained. The agar is scraped off the plates and pressed through sterile gauze (8 layers), the liquid thereby obtained being subsequently filtered through an asbestos bacterial filter. The filtrate, whose final pH should not exceed 7.6, is stable for long periods at 2-4°C, but should be tested at frequent intervals for potency as indicated below. The quality of the peptone used in the media is a most important factor in preparing a potent filtrate. Different brands of available peptone should be compared, and that found to be most effective should be desiccated and sealed in vacuo. It may then be stored at 4°C.

Potency test

The test for potency of the filtrate is carried out by mixing normal serum with the filtrate in a proportion of 1 of serum to 4 of filtrate and incubating overnight at 37°C, after which the mixtures are heated for 1 hour at 56°C. Sera from ferrets, rabbits, or fowls are all suitable but require separate titrations.

With the treated material, haemagglutination-inhibition tests are set up against two strains of virus. The viruses\textsuperscript{1} are selected so that one is mouse-adapted and yet retains its sensitivity to inhibitor, and the other a recently isolated influenza A strain of the FM1 group, cultivated only in eggs, which is also highly susceptible to inhibitor.

The actual test is carried out by mixing one volume of test virus containing 4 haemagglutinating units with 2 volumes of the undiluted serum-filtrate mixture. The whole is placed at 2-4°C for half an hour, and one volume of 2% fowl red-cells in saline is then added. The final dilution of serum in this mixture is 1/10. At this strength of serum no inhibition of haemagglutination should be obtained with either virus. Dilutions of the serum-filtrate mixture are usually prepared and tested at the same time as an indication of the relative potency of weak filtrates. The procedure of mixing virus and serum together half an hour before adding the red cells greatly increases the sensitivity of the test for inhibitor.

Because some batches of cholera filtrate may contain excessive amounts of calcium, which will stabilize to heat the red-cell receptor-destroying enzyme, it is necessary to include appropriate controls to ensure that the period of heating at 56°C has, in fact, produced inactivation. If not, the batch should be discarded.

\textsuperscript{1} Strains of virus which have proved satisfactory in the above test are the mouse-adapted strain A/Nederland 1941 and the egg-adapted strain A-Barrett/(England)/1947.
B. METHOD OF TREATMENT OF SERA WITH CARBON DIOXIDE FOR REMOVING NON-SPECIFIC INHIBITORS OF INFLUENZA VIRUS

The treatment of sera with carbon dioxide is carried out as follows:

The serum is first diluted 1/10 with distilled water. Carbon-dioxide gas is then passed through the serum until an intense turbidity appears (formation of a precipitate). This takes approximately 5-10 minutes.

The serum is centrifuged for 10 minutes at 1500-2000 r.p.m. and 0.1 ml of 8.5% NaCl solution is added to each 0.9 ml of the supernatant fluid to restore the physiological concentration of salts.

FIG. 1. TYPES OF DROPPING FUNNEL USED INSTEAD OF KIPP'S APPARATUS FOR OBTAINING CO₂

* Reproduced from *Vop. Virusol.*, 1956, 7, 22, by kind permission

To prepare the carbon dioxide the usual Kipp’s apparatus can be used. Alternatively, a simpler apparatus may be used as shown in Fig. 1. Marble may be used instead of amorphous CaCO₃. The HCl is used in a dilution of 1/3 or 1/4 to avoid contamination of the serum by the acid gas.

Carbon dioxide can be also used in the form of “dry-ice” (frozen carbon dioxide), in which case small pieces of dry-ice should be put into the diluted serum.

All these methods of treatment of sera with carbon dioxide result in precipitation of non-specific inhibitors (both α and β), the specific antibodies remaining in the supernatant fluid. Removal of inhibitors occurs
only after preliminary dilution of the serum with distilled water. The presence of physiological concentration of salts prevents the formation of the precipitate.

C. METHOD OF TREATMENT OF SERA WITH PERIODATE FOR REMOVAL OF NON-SPECIFIC INHIBITORS

It has been clearly established that solutions of M/90 potassium periodate will destroy inhibitor. This procedure can be carried out as follows: to one volume of serum add three volumes of the periodate, mix well and incubate for at least 15 minutes at room temperature. At the end of this time add a volume of 1% glycerol-saline equal to that of the periodate. This step neutralizes any excess periodate. It is important that the solution of periodate be freshly prepared and that it be not heated to put in the solution. Furthermore, potassium periodate is generally more satisfactory than is sodium periodate.

D. TECHNIQUES RECOMMENDED FOR STANDARD DIAGNOSTIC PROCEDURES

1. Complement-fixation test

The following technique is based upon the procedure recommended by Hoyle, but the antigens are prepared from infected eggs instead of mouse lungs as in the original method.

1.1 Reagents

1.1.1 Antigens

The antigens are prepared in one of two ways, both of which have been found satisfactory in different laboratories.

The PR8 or WS strain of influenza A and the Lee strain of influenza B are used in both methods.

Method A: Chick embryos are inoculated allantoically after 10 or 11 days' incubation with 0.1-ml amounts of a $10^5$ or $10^4$ dilution of infected allantoic fluid. After 42-48 hours' further incubation at 35°C, the membranes are removed and suspended in physiological saline (1 ml per membrane), frozen rapidly in a mixture of alcohol and dry-ice, and

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allowed to thaw out slowly at room temperature. The freezing and thawing are carried out three times, and the suspension is then centrifuged at 3000 r.p.m. for 15 minutes. The supernatant fluid is removed, and 2% chloroform is added to it. This is shaken up thoroughly and allowed to stand overnight at 4°C. The suspension is again centrifuged and the supernatant is removed and used as the source of soluble antigen. It can be stored at 4°C, but it is recommended that the material should be dried from the frozen state for storage for periods longer than one month.

Method B: Seed. Nine-day embryonated eggs are inoculated with 0.4-ml amounts of a $10^4$ dilution of virus-infected allantoic fluid. After further incubation at 35°C for 40-44 hours, the eggs are chilled and the allantoic fluids are removed. The fluids, which should titre between 1/640 and 1/1280 by the haemagglutination method, are used immediately or stored for an indefinite period in glass-sealed ampoules in the dry-ice box at $-70^°$C.

Preparation of antigen. 0.5-ml amounts of the undiluted allantoic fluid described above are inoculated into the allantoic cavity of 11-day embryonated eggs. After 6 hours' incubation at 35°C, the eggs are opened and the chorio-allantoic membranes are removed. These are thoroughly washed in three separate vessels of physiological saline solution, drained on blotting-paper, and weighed. They are then ground in a Waring blender for 3 minutes with an equal weight of physiological saline solution to give a 1/2 dilution. The material is then centrifuged at 3000 r.p.m. for 10 minutes to sediment the tissue, and the supernatant is removed. Infectivity is destroyed by minimal ultra-violet irradiation, the exact conditions for which will need to be worked out for the individual lamp. Tests for infectivity are made in eggs, using antigen undiluted and diluted $10^1$ and $10^3$ to obviate interference effects.

Normal control-antigens are prepared from uninoculated 11-day embryonated eggs in an identical manner.

These preparations are stable for an indefinite period when stored at $-20^°$C or at $4^°$C.

1.1.2 Sera

These are inactivated at $56^°$C for 30 minutes. If not used at once, either they should be stored at $-20^°$C, or 0.08% sodium azide should be added and the sera should be kept in the refrigerator ($4^°$C).

1.1.3 Complement

Poolled guinea-pig serum is stored at $-70^°$C. At this temperature the complement maintains its titre over a long period.
In the absence of storage facilities, complement may be preserved by the boric-acid sorbitol azide method of Richardson: 1

Solution A
Boric acid (H₃BO₃) 1.55 g
Saturated NaCl to 100 ml

Solution B
Sorbitol (C₆H₁₂O₆, ½ H₂O) 9.55 g
Sodium azide (NaN₃) 0.81 g
Saturated NaCl to 100 ml

Solution C
Sodium azide 0.81 g
Saturated NaCl to 100 ml

To each 8 ml of serum add 1 ml of solution A and 0.5 ml each of B and C. Store in the refrigerator, and for use dilute 1/8 with distilled water; this gives a 1/10 dilution of complement.

1.1.4 Diluting fluid

Physiological saline is used in connexion with the above antigens, though other types of antigens prepared from eggs may require the use of buffers such as that recommended by Mayer et al. 2

1.1.5 Haemolytic system

A 2% suspension of packed sheep cells is used, and to this is added an equal volume of haemolysin dilution containing 10 Minimal Haemolytic Doses (MHD).

1.2 Titration of complement

A series of tubes is set up containing 0.2 ml of complement dilution (1/10, 1/15, 1/20, 1/25, 1/30, 1/35, 1/40, 1/50, 1/60, 1/70, 1/80, 1/90, 1/100). To each tube are added 0.4 ml of saline and 0.4 ml of sensitized red blood-cells. The tubes are incubated in the water-bath at 37°C for 30 minutes. A reading is made of the highest dilution giving 100% haemolysis, and also of the highest dilution giving 50% haemolysis (made by throwing down the cells by centrifugation and matching the supernatant fluid against a 1/2 dilution of a tube giving complete haemolysis). For use in complement-fixation tests, the complement is diluted so as to contain in unit volume 2 ½ MHD by the 100% haemolysis reading or 4 ½ MHD by the 50% haemolysis reading. These two readings usually correspond

1 Richardson, G. M. (1941) Lancet, 2, 696
exactly; when any discrepancy occurs, the 50% haemolysis titre is the more accurate.

Preserved complement retains its titre unchanged for some weeks. After it has once been accurately titrated, it is better to use the same dilution each day rather than to titrate it afresh every time a test is done. In other words, it is better always to use the same amount of complement and to ignore minor variations in sensitivity of the haemolytic system.

1.3 *Titration of antigen*

Each batch of liquid antigen prepared is titrated against an excess of a known positive serum. Tubes are set up containing 0.2 ml of antigen dilution (1/1, 1/2, 1/4, 1/8, 1/16, 1/32), 0.2 ml of complement, and 0.2 ml of serum dilution (e.g., serum titre 1/64 would be used diluted 1/8). Tubes are incubated for 1 hour in the water-bath at 37°C; 0.4 ml of sensitized cells are added; the tubes are incubated again for 30 minutes, the cells allowed to settle, and readings made. Any tube showing less than 50% haemolysis is regarded as positive; 50% haemolysis or more is read as negative.

This test gives a reading of the maximal antigen titre. The optimal amount of antigen for use in serum titrations is three times the maximal titre, but in practice four times the maximal titre is used, as it is better to have a slight excess of antigen rather than too little.

1.4 *Standard test for titration of antibody in serum*

Each tube in the test contains 0.2 ml of antigen, 0.2 ml of complement, and 0.2 ml of serum dilution.

For normal sera, serum dilutions of 1/2, 1/4, 1/8, 1/16 are used, and for convalescent sera, dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256.

In addition, a serum control is set up in which a serum dilution of 1/2 is used and the antigen is replaced by saline.

A fixation period of 1 hour at 37°C is used, the tubes being incubated in the water-bath and shaken after 30 minutes. After 1 hour, 0.4 ml of sensitized cells are added, the tubes are incubated for 30 minutes, the cells allowed to settle, and readings made.

Tubes showing less than 50% haemolysis are read as positive, and 50% haemolysis or more as negative, except in the case of the 1/2 dilution of serum where a reading of exactly 50% haemolysis is read as doubtful.

Sera may therefore be regarded as negative, doubtful (1/2), or positive at titres ranging from 1/2 to 1/256.
1.5 Small-volume and micro-tests

The standard test requires the use of large volumes of antigen. When testing numerous sera, it may be desirable to use smaller volumes of reagents.

<table>
<thead>
<tr>
<th>Standard test</th>
<th>Small-volume test</th>
<th>Micro-test</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
<td>0.2 ml</td>
<td>0.1 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Complement</td>
<td>0.2 ml</td>
<td>0.1 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Serum dilution</td>
<td>0.2 ml</td>
<td>0.1 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Sensitized cells</td>
<td>0.4 ml</td>
<td>0.2 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>1.0 ml</td>
<td>0.5 ml</td>
<td>0.25 ml</td>
</tr>
</tbody>
</table>

For the micro-test some form of constant-volume pipette is necessary to deliver 0.05 ml. With care the micro-method can be made almost as accurate as the standard test.

The test using 0.1-ml volumes of antigen, complement and serum, and 0.2 ml of sensitized cells, has been found both satisfactory and economical in reagents and can be recommended.

2. Haemagglutination-inhibition test

2.1 Materials required

(1) Saline solution (0.85% NaCl in distilled water)
(2) 2% sodium citrate (in saline solution)
(3) 1-ml serological pipettes, graduated in 0.01 ml
(4) Kahn tubes (12 × 75 mm) with evenly rounded bottoms, and suitable racks
(5) Standard erythrocyte suspension (see section 2.2, below)
(6) Standard antigens (see section 2.3, below)
(7) Standard antisera (see section 2.4, below)

2.2 Standard erythrocyte suspension

The standard suspension should consist of 0.5% chicken erythrocytes in saline solution (human “group O” erythrocytes may be substituted if desired).

Obtain blood from wing vein or from heart and add immediately to 0.2 volume of 2% sodium citrate. The citrated blood may be preserved for not longer than one week at 4°C.

The standard erythrocyte suspension is prepared fresh for each day’s work in the following manner. Wash approximately 10 ml of the citrated blood three times, each time with approximately 50 ml of saline solution. After the third washing, suspend the cells in approximately 10 ml of saline solution and transfer to a 15-ml conical, graduated, centrifuge tube.
The tube should be held in a 50-ml centrifuge cup (No. 320, International) employing a reducing cap (No. 312, International) to adapt the cup to the 15-ml tube.

Centrifuge in a horizontal centrifuge at a gravity force of 3200 for 10 minutes. Determine the volume of cells by direct reading of the tube graduations; discard the supernatant fluid; and then suspend the cells in enough saline to make a 0.5% suspension (199 volumes).

2.3 Standard antigens

For the present, it is recommended that antigens should be prepared from type A PR8 and FM1, and Lee or Bon strains of B virus. Each should be from a single pool of allantoic fluid. The pooled allantoic fluid should be from eggs inoculated with seed material selected for the purpose and harvested from eggs containing embryos not older than 12 days.

In order to lessen the formation of precipitate on standing, the pooled allantoic fluids should be diluted 1/4 in saline solution and should contain 1/10 000 Merthiolate as a preservative. This 1/4 dilution containing 1/10 000 Merthiolate is the "stock antigen" and should titre not less than 1/64. It must be stored in a refrigerator at approximately 4°C.

2.3.1 Haemagglutinating unit

The haemagglutinating unit is contained in 0.25 ml of the highest dilution of stock antigen which completely agglutinates the standard chicken erythrocyte suspension. The unit should be determined as follows:

(1) Remove 1.0 ml of stock antigen from the container, and dilute with 3.0 ml of saline solution. This is the "working dilution".

(2) Set up two rows of Kahn tubes, 10 tubes in each row. Place 1.0-ml amounts of saline solution in each tube of the front row. Then prepare and dispense serial twofold dilutions as follows:

With a 1-ml pipette, add 1.0 ml of the working dilution to the first tube of the first row; discard the pipette. Mix well with a fresh pipette, and transfer 1.0 ml of the mixture to the second tube of the first row, and 0.25 ml to the first tube of the back row; discard the pipette. With a fresh pipette, mix the contents of the second tube of the first row, and then transfer 1.0 ml of the mixture to the third tube of the first and 0.25 ml to the second tube of the back row. Continue through the series, until each tube of the back row contains 0.25 ml of serial twofold dilutions. Discard front row of tubes.

(3) To each tube, add 0.25 ml of saline solution (to bring the volume up to 0.5 ml).

(4) Add 0.5 ml of the 0.5% chicken erythrocyte suspension to all tubes.
(5) Include an erythrocyte control of 0.5 ml of saline solution and 0.5 ml of the erythrocyte suspension.

(6) Mix well by shaking.

(7) Incubate at 22°-24°C; after 60 minutes, read the results on the basis of the pattern. (In hot countries where room temperature is over 24°C (75°F approximately) the alternative of an ice-box technique (0°-4°C) should be permitted, results to be read after 2 hours, immediately after removal from the cold.)

The 0.25-ml quantity of the highest dilution of the working dilution which completely agglutinates the standard erythrocyte suspension is one unit (i.e., contains one unit of activity).

2.3.2 Test antigen

The test antigen should contain four haemagglutinating units of virus. This is provided by diluting the working dilution so that it will be four times more concentrated than the highest dilution of the working dilution which showed complete haemagglutination in the above test.

**Example:** If the greatest dilution of the working dilution which completely agglutinated the erythrocyte suspension was 1/64 (6th twofold dilution), the desired dilution of the working dilution is 64/4, that is, 1 part working dilution, plus 15 parts of saline solution.

The antigen so prepared (test antigen) should be tested in the manner described above (section 2.3.1, paragraphs 2, 3, and 4, page 46) in order to ensure that it contains 4 haemagglutinating units. Complete agglutination of erythrocytes must be present in the first two tubes of the series, and the third tube must show less than complete haemagglutination.

2.4 Standard antisera

Sera with known inhibitory properties against each of the test antigens should be included in the test.

2.5 Test for haemagglutination-inhibiting antibody content of sera

Pairs of sera are absolutely necessary for diagnostic tests. The antibody titres of unknown sera are determined by comparing in the same test the pairs of sera, and known antisera, against the standard antigens in the following manner:

(1) All test sera should be inactivated at 56°C for 30 minutes.

(2) For each serum to be tested, set up four rows of Kahn tubes, 10 tubes in each row. Add 1.0 ml of saline solution to each tube of the first row. To the first tube of the first row, add 1.0 ml of a 1/8 dilution
of the serum (0.2 ml of serum plus 1.4 ml of saline solution). Mix well, and transfer 1.0 ml of the mixture to the second tube of the first row, using a 1-ml pipette. Mix well, and transfer 1.0 ml of the mixture to the third tube of the first row. Continue through all ten twofold serial dilutions of this row. With the same pipette, now distribute 0.25 ml of the 10th twofold dilution to each of the last tubes of the other three rows. Then transfer 0.25 ml of the 9th dilution to each of the corresponding tubes of the other three rows. Continue with the 8th, 7th, and other dilutions until all have been distributed. The tubes in the front row are discarded and are not used in the final test. If it is desired to use two strains of influenza virus B, the volumes indicated will need to be modified accordingly.

(3) Prepare suitable dilutions of the known antisera.

(4) After the serum dilutions have been prepared, add 0.25 ml amounts of each of the test antigens to each tube of the appropriate row.

(5) To the serum-virus mixtures, add 0.5 ml of the 0.5% erythrocyte suspension.

(6) Shake well and examine to be sure that all cells are well suspended.

(7) Incubate at 22°-24°C for 60 minutes, and then read on the basis of the pattern of the settled cells.

The titre is expressed as the highest initial dilution of serum which effects complete inhibition of agglutination.

The test antigens must be re-titrated at the same time that the haemagglutination-inhibition tests are performed, in the manner described for the re-titration of the test antigen in section 2.3.1, page 46; again, complete agglutination should occur in the first two tubes of the series of twofold dilutions, and the third tube should show less than complete agglutination.

Titration of virus and antisera may be modified to some extent according to individual preferences. The use of individual pipettes in preparing serial dilutions of antigens or antisera is not necessary, provided adequate care is taken in the use of a single pipette.

3. Haemagglutination-inhibition test in influenza C infections

The diagnostic haemagglutination-inhibition test for influenza C has not been entirely satisfactory in all hands, primarily because of the difficulty in preparing antigen in the amniotic cavity. The test has, however, met with success in some laboratories; and a procedure found satisfactory is given below:

Influenza C virus grows poorly in the allantoic cavity of embryonated eggs, but the virus can be propagated in high titre in the amniotic sac. The 1233 strain of virus is preferred since it has been found to give
higher titres than some of the other strains. Embryonated eggs, incubated for 10 days at 39°C, are inoculated into the amniotic cavity with 0.1 ml of a 10^2 dilution of suitable seed virus preparation. After further incubation at 35°C for 26-30 hours, the eggs are chilled and the amniotic fluids are harvested; these materials are used immediately or are stored in 0.5-ml amounts of lyophilized form.

Antiserum to influenza C virus can be prepared in roosters using the basic technique outlined above. However, a single intravenous injection of 2.5 ml, and 2.5 ml given intraperitoneally, will usually suffice. Haemagglutination-inhibition titres as high as 1/25 600 have been obtained. Since all sera tested to date, both human and animal, have appeared to be free of non-specific inhibitors, cholera filtrate should not be used.

Haemagglutination and haemagglutination-inhibition titrations are performed by a modification of the standard haemagglutination-inhibition technique described in this annex. Because influenza C virus rapidly elutes from red cells at room temperature, titrations of serum and antigen must be made in the cold (4°C). The virus titrations are read after 75-90 minutes' incubation, and the haemagglutination-inhibition tests after 60-70 minutes'. Human red cells are employed in the tests.

4. Modification of the haemagglutination-inhibition test for use with plastic plates

Titrations of virus and of sera can be carried out in plastic plates instead of tubes.

Serial twofold dilutions (0.25 ml^1) of virus are prepared in physiological saline, and equal volumes of 0.5% fowl cells are added. Readings are made by the pattern method and the end-point is taken as partial (50%) agglutination. The 50% end-point is determined by observing the pattern made by a 1/2 dilution of virus showing just complete agglutination. Where partial (50%) agglutination does not show in any dilution, the end-point is determined by interpolation.

For titration of serum antibodies, serial dilutions of serum (0.25 ml) are made in saline and an equal volume of red cells added, followed immediately by a third volume of antigen. The antigen is made up to contain 8 partial agglutinating doses of virus per 0.25 ml, and a control test of the concentration of virus actually present is carried out along with the anti-haemagglutinin test. Partial agglutination is taken as the end-point, and interpolations for the end-point are again made where necessary.

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1 The volumes given are those used in conjunction with the plates as distributed by the World Influenza Centre. Plates with different-sized cups will require different volumes.
Annex 4

METHODS AND PROCEDURES FOR USE OF COMPLEMENT-FIXATION TECHNIQUE IN TYPE- AND STRAIN-SPECIFIC DIAGNOSIS OF INFLUENZA

The existence of two kinds of complement-fixing antigens of influenza virus is well established. The soluble (S) antigen is predominantly found in infected tissues (free S) but it is also present within the virus particle (internal S). The virus (V) antigen is closely linked with the haemagglutinating component, if not identical with it. The S antigens are type-specific; i.e., they are identical for all strains of one type. The V antigens are strain-specific; i.e., they differ to a greater or lesser extent depending upon the year of isolation of the strain, in analogy with the differences measured by the haemagglutination-inhibition technique.

In order to obtain valid results in complement-fixation tests concerning various aspects of diagnosis it is obviously essential to prepare reliable reagents; that is, V antigens free of S and S antigens devoid of V and, correspondingly anti-V sera lacking anti-S and anti-S sera without anti-V. It is now possible to produce these reagents by relatively simple means. The specific sera are helpful in the rapid identification of new isolates of influenza virus as to type and in the determination of antigenic patterns of strains. Pure S and V antigens are of advantage in the type- and strain-specific serodiagnosis of the disease.

In the following sections the methods of preparation of the reagents and their application to various diagnostic problems will be presented.

Preparation of Reagents

V antigens

For in vitro antigens, virus particles, separated from free or external S, are satisfactory. The internal S is not available for interaction with antibodies. For the production of anti-V sera in guinea-pigs, however, it is preferable to employ V antigens from which the internal S has been removed.
V antigens for in vitro tests

For this purpose suspensions of virus particles are used which are derived from infected allantoic fluids by two cycles of adsorption on to and elution from chicken red cells. Bloody allantoic fluids are harvested from infected chick-embryos shortly after removal from the incubator by tearing the allantoic membranes and permitting free bleeding into the allantoic cavity. The bloody fluids are then immediately placed in an ice-bath for one hour to allow for adsorption of the virus on to the red cells. As an alternative, clear allantoic fluids may be harvested from chilled eggs and washed and packed erythrocytes added to the ice-cold fluids to form a 5% suspension. After the adsorption period the cells are sedimented by centrifugation in the cold, washed once with ice-cold M/100 phosphate-buffered saline solution (BSS) at a pH of 7.0 and resuspended in BSS containing 10 units of receptor-destroying enzyme (RDE), 500 units of penicillin and 100 μg of streptomycin per ml. For each egg harvested 1 ml of BSS is employed. Elution of the virus from the red cells is permitted to take place in a waterbath at 37°C for 2-2½ hours. After removal of the red cells the eluates, constituting sixfold to eightfold concentrations of the virus, are subjected to second cycles of adsorption and elution as described, except that the volumes of BSS used for resuspension of the cells should be adjusted so that the final eluates will contain a minimum of 2560 haemagglutinating (HA) units per ml. For determination of the HA units, 0.4 ml of virus dilution is mixed with 0.2 ml of a 1% suspension of chicken red cells. The HA titre so obtained, multiplied by 2.5, yields the number of HA units per ml. If other proportions of virus and red cells are employed in the HA test, one HA unit may represent different amounts of virus and the unitage needed for satisfactory V antigen preparations would have to be adjusted accordingly.

The V antigens so obtained are generally free of detectable S. If found, a third cycle of adsorption on to and elution from red cells is required for its removal.

V antigens for immunization of guinea-pigs

Virus suspensions obtained after one cycle of adsorption on to and elution from red cells (see above), and containing at least 5120 HA units/ml, are mixed at room temperature with half a volume of anaesthetic ether and agitated continuously for one hour on a magnetic stirrer so that the ether is kept well interspersed with the virus suspension. The mixtures are then transferred to separating funnels and the aqueous phases are withdrawn. Residual ether is best removed by bubbling nitrogen through the materials. The treatment with ether liberates the internal S, and the HA components may now be separated from the S fraction by adsorption on to and elution from chicken red cells according to the technique described
above. The treatment with ether reduces the HA activity of certain strains when tested with chicken red cells, whereas agglutination of guinea-pig erythrocytes is usually increased by a factor of 4. In these cases, the use of guinea-pig instead of chicken red cells is recommended for better yields. A second cycle of adsorption and elution is usually needed to free the HA fractions of detectable S antigen. This is best carried out in the absence of RDE, since on immunization with the eluates guinea-pigs may on occasion form antibodies to this enzyme. In this case the elution period has to be prolonged to up to 6 hours at 37°C in order to recover most of the HA components.

A few virus particles may escape the action of ether and remain infectious. It is recommended, therefore, that the preparation be exposed to ultraviolet light in order to ensure complete inactivation, since an infectious process may result in production of some anti-S. Irradiation of the material in 15-20 ml volumes in open Petri dishes under constant slight agitation for three minutes by a General Electric Germicidal Lamp (or its equivalent) at a distance of 7 inches, or about 18 cm, has been found satisfactory for this purpose. Before use, the preparations are tested for the presence of infectious virus by two blind passages in chick-embryos.

\[S\text{ antigens}\]

\textbf{S antigens may be derived from infected allantoic membranes or fluids (free S) or from virus particles (internal S). The richest source of S antigen is infected chorio-allantoic membrane. While all three kinds of preparation are satisfactory for \textit{in vitro} tests, for immunisation of guinea-pigs internal S is preferred because of its relative purity.}\]

\[S\text{ antigens for in vitro tests}\]

\textbf{Infected chorio-allantoic membranes.} These are collected 24-48 hours after inoculation of the chick-embryos, the optimal time of harvest depending upon the dose of virus inoculated.\textsuperscript{4} A 20%-40% suspension of the tissue in BSS, clarified by low-speed centrifugation, contains as a rule S antigen in excess of V and thus may be used for serological tests. However, in order to avoid all possible interaction of the V antigen also present, it is desirable to remove all HA activity by high-speed centrifugation or exhaustive adsorption of the suspension with chicken erythrocytes, or by both processes.

\textbf{Infected allantoic fluids.} Such fluids, particularly when collected 48-72 hours after inoculation of the eggs, also contain free S.\textsuperscript{4} In this case, the V antigens exceed, as a rule, the concentration of S. Such preparations may serve as reliable S antigens only when the virus particles are removed
by high-speed centrifugation or exhaustive adsorption with chicken red cells.

Internal S antigen. Internal S antigen is liberated from virus particles by exposure to ether as described earlier. In this case, adsorption with chicken red cells is often not sufficient because as a result of ether treatment the affinity of the haemagglutinating components for chicken red cells may be reduced, whereas that for guinea-pig erythrocytes is generally increased.\(^7\) For this reason S preparations derived from certain strains of virus may require adsorption with guinea-pig red cells in order to remove all V antigen.

\textit{S antigen for immunization of guinea-pigs}

For this purpose S antigen is employed as an intra-abdominal booster injection following intranasal inoculation of active virus (see below). Any one of the S preparations described above exerts the desired booster effect, but in order to minimize anti-V responses the S preparations must be exhaustively adsorbed with red cells. The internal S antigen, liberated from virus particles by ether, constitutes the purest and therefore the most desirable preparation for this purpose.

\textit{Anti-V sera}

Guinea-pigs are injected intra-abdominally with the HA fraction derived from ether-treated virus particles which have been proved to be non-infectious by two consecutive blind passages in chick-embryos. The preparation should contain 2560-5120 HA units per ml as measured with chicken red cells, or when this activity has been affected by ether, with guinea-pig red cells. Three doses of 1 ml each are usually sufficient to evoke a satisfactory anti-V response. If preparations of lesser HA activity are the only ones available larger volumes may be injected. The first two doses are best given 5-7 days apart, followed by the third dose 3 weeks later. The sera collected one week after the last injection generally reveal high anti-V titres (1: 64 or greater) and no anti-S (<1: 4).\(^1\) Rare sera showing slight anti-S activity are discarded. Occasionally a fourth dose may be required to elicit satisfactory antibody responses but further injections, as a rule, fail to improve the results. Other dosage schedules, such as three injections at 4-5-day intervals and bleeding 12-18 days later, have also given suitable anti-V responses. The sera are inactivated at 56\(^\circ\)C for 30 minutes, absorbed with sheep cells and stored preferably in the frozen state.

A few animals may form antibodies to RDE present in the final eluate employed for immunization. This is more evident when volumes in excess
of 1 ml are injected. This response is eliminated by avoiding the use of RDE in the final elution procedure.

**Anti-S sera**

Guinea-pigs are inoculated under light ether anesthesia with infected allantoic fluid, diluted so as to contain about 10⁶ or fewer chick-embryo-infectious doses, 0.1 ml being dropped into each nostril. After 5-8 weeks, when the antibodies evoked by the infection, especially the anti-V, are expected to have decreased to subdetectable levels, an intra-abdominal injection of 1 ml of S antigen is administered. The antigen preferably to be used for the booster dose is the internal S antigen derived from virus particles by exposure to ether. If the intranasal inoculum consists of virus of one subtype (e.g., an Al strain) and the S antigen is derived from another subtype (e.g., an A strain) only the antibodies to the common S antigen are boosted. Under these conditions about half the animals will reveal 7-10 days after the intra-abdominal injection anti-S titres of 1:64 or greater and no detectable anti-V. Any V antigen present in the S preparation is generally insufficient to stimulate homologous anti-V and incapable of recalling the heterologous anti-V of the strain used for infection. The sera are inactivated and stored as described above.

If the strains used for the intranasal infection and for preparation of the S antigen for the booster injection are identical or closely related, the sera obtained reveal both anti-V and anti-S in high titres. Such sera can be used for detection of S antigen in heterologous, homotypic virus preparations as long as the anti-V in the serum bears no relationship to the V antigen under test.

**Test Procedures**

Numerous modifications of the complement-fixation test are in practice in different laboratories. As long as the standardization procedures for the various reagents are rigid there is no particular reason to prefer one or the other method. For this reason no technical details concerning the test itself will be given below, and the presentation will be restricted essentially to principles which should be part of all methods.

**Standardization of complement**

Since the various viral preparations derived from chick-embryos often exhibit pro-complementary activity (or on occasion anti-complementary effects), titration of complement should be performed always in the presence of representative antigens in the test dose to be employed. This
will ensure accurate dosage of complement for the various antigens under test, whether two full units or any other unitage is desired.

Identification of isolates as to type and antigenic patterns

*Tests for potency and specificity of anti-V and anti-S sera*

Since zone phenomena are common in the interaction of influenza antigens with their respective antibodies, the sera to be used for identification of isolates have to be assayed for potency and specificity in block titrations, also referred to as two-way, box, or optimal titrations. Duplicate sets of falling twofold dilutions of serum are tested against serial twofold dilutions of (a) the homologous V and (b) the homotypic S antigens. A suitable anti-V serum should react in high titre (1:64 or more) with the homologous V antigen but not at all with S, and the anti-S serum should react in high titre with homotypic S but not at all with the V antigens of the strains involved in the production of the anti-S sera.

*Standardization of antigens*

*Reference antigens.* The block-titration technique described above serves to standardize the reference V and S antigen with respect to their purity and potency. The optimal unit of V or S antigen is the highest dilution of antigen giving maximal homologous serum titres. It should be noted that the unit determined with appropriate human sera may require twice the amount of antigen measured with guinea-pig sera.

*New isolates.* It is not always feasible to prepare V antigens from new isolates according to the method described above for *in vitro* tests and to standardize them by the same techniques as reference antigens. The delay caused by their preparation and in obtaining specific antisera to them is particularly contra-indicated in times of an epidemic. Fortunately, for purposes of strain identification, antigens may be used which contain S. The S present will not interfere in the reaction provided the anti-V sera used for assay are free of anti-S. Thus infected allantoic or amniotic fluids may serve as V antigens. In the absence of specific antisera, the V unitage of either elementary body suspensions or infected allantoic fluids may be estimated from the HA titres, since one V antigen unit corresponds to 16 HA units under the conditions of testing referred to above.

* Determination of type of isolates*

For this purpose allantoic or amniotic membrane suspensions of HA-positive eggs in the first few passages form a better source of S than the
respective fluids, unless the HA titres of these are so high as to suggest that S antigen may also be present in detectable quantities. Suspensions of the tissues, prepared as described earlier, are tested against 4 or more antibody units of anti-S sera of the various types. Known S antigens serve as controls for the sera.\textsuperscript{10}

**Determination of antigenic composition of isolates**

The techniques to be employed vary depending upon whether one deals with a member of an established subtype, which has been in circulation for several years, or with a new antigenic variant, representing the first member of a new subtype.\textsuperscript{9}

**Strains of an established subtype**

The new isolate, either in the form of infected allantoic fluid or, if possible, in the form of a suspension of virus particles obtained by two cycles of adsorption on to and elution from red cells, is first screened against various anti-V sera prepared with prototype strains of homotypic viruses. The sera are used in dilutions of 1:4 or 1:8 and the dose of antigen should represent preferably 8 or more V units; i.e., 128 or more HA units per test volume. The sera giving a positive complement-fixation reaction are then titrated in falling twofold dilutions against the isolate antigen. The titres obtained are compared with the titres of the anti-V sera with their homologous antigens. If one of the anti-sera reveals similar titres with the isolate and its homologous V antigen, the new agent is assumed to be closely related with respect to the dominant antigens of the strain employed for production of that serum. The incidence and degree of cross-reactions of these two viruses with other anti-V sera will indicate how closely they resemble each other with respect to minor antigenic components. If none of the reactive sera reveals matching titres with the isolate and their homologous V antigen some change in the dominant antigen must have occurred and antisera to the new virus should be prepared to evaluate the extent of that change.

If none of the anti-V sera shows a reaction, yet the type was clearly established, a new subtype may have arisen and such isolates should receive therefore immediate, intensive study in an appropriate laboratory.

**New subtype**

With the advent of a new subtype, such as the recent appearance of A2, the identification in the first major epidemic can be restricted to a screening procedure, using infected allantoic or amniotic fluid as source
of antigen and anti-V sera against prototype viruses. The anti-V sera may be used in a dilution containing 4 homologous anti-V units in order to avoid the rare, insignificant cross-reactions that might occur between members of two subtypes. In this fashion only the serum to the new subtype will give a positive reaction. If the HA titre of the virus preparation is low, as often experienced in early passages, the volume tested in the complement-fixation test may be increased up to sixfold without interfering with the reaction. Thus, if the usual volume of antigen employed is 0.1 ml, up to 0.6 can be used without changes in the volumes of the other reagents. The test may then be read after settling of the red cells. Absence of any haemolysis serves to identify the positive reaction. Under such conditions, an allantoic fluid having as few as 30-40 HA units per ml can be used, since the increased volumes will still contain 16 units, which correspond to one V unit.

Serodiagnosis

The serological diagnosis of influenza during an epidemic, once the offending strain has been established, may well be restricted to the use of S antigen of the corresponding type. However, under certain conditions, such as the appearance of a new subtype, strain-specific serological tests may be of considerable aid. In the past these were restricted to haemagglutination-inhibition tests but now the complement-fixation technique may also be employed, using strain-specific V antigens.

For standardization of V and S antigens and reference anti-V and anti-S sera, the reader is referred above to the section on identification of isolates as to type and antigenic patterns.

Type-specific serodiagnosis

For this purpose acute-stage and convalescent sera are titrated against 2-4 optimal dilutions of S antigen. A fourfold or greater rise in anti-S of one type is considered evidence of infection with a strain of that type. Anti-S responses are the rule except in very young children (under two years of age), where no anti-S may be found.

Note. In many laboratories allantoic fluid infected with any one strain of given types or crude allantoic membrane suspensions have been used for serodiagnosis with apparently satisfactory results. However, both types of preparation also contain V antigen. In the allantoic fluids, V antigen is generally found in excess of S, and in the membranes the reverse is true. It is likely that the allantoic fluid antigens, even if old strains are employed, detect essentially a recall of antibodies to V antigens which may be present in minor or even subdetectable concentrations in the infecting virus. The diagnosis consequently might be missed in persons who had few previous experiences with influenza (younger age-groups) and in individuals who already possessed that particular anti-V prior to illness and in whom the stimulus provided by the infection was sufficient to increase the titre.
With the crude membrane suspensions it is likely that mainly anti-S responses are measured but the low levels of V, if not diluted out by restriction of S to 2 optimal units, may interfere with arriving at a diagnosis under the conditions described above.

For these reasons the S antigens for in vitro tests described above would seem to be preferable, particularly since the additional step in their preparation, i.e., their adsorption with chicken red cells for the removal of V, is simple and feasible in any laboratory.

Strain-specific serodiagnosis

For this purpose V antigen, preferably of the current strain, or at least a close relative, is required. This is prepared according to the method described above for in vitro tests. The acute and convalescent sera are titrated against 2-4 optimal units. If the acute-stage serum is obtained early enough a significant rise in antibodies may be seen in the convalescent sera of practically all patients, including infants, where anti-S responses may not be elicited. In the case of a first infection with a new subtype, such as A2, the anti-V response may be delayed and convalescent sera obtained 10-14 days after onset may still be negative, whereas later bleedings yield high titres of antibodies.

As is known from experience with the haemagglutination-inhibition test, antibodies to predominant V antigens of other strains may also frequently rise. These represent a recall of antibodies, elicited by the booster effect of earlier antigens present in the infecting virus in minor or even undetectable concentrations.

The use of the "current" V antigen in serodiagnosis has the following advantages: nearly all patients respond with anti-V, usually from <1 : 4 in the early acute stage to > 1 : 64 in convalescent stages; it may still reveal a diagnostically significant rise in antibodies when the acute-stage serum is taken relatively late after onset so that anti-S is already high as a result of a recall and does not significantly increase thereafter; and it reveals an antibody response in infants where anti-S may fail to develop.

Strain-specific antibody responses following vaccination

The techniques employed for strain-specific serodiagnosis can be applied to assay of anti-V responses following vaccination. The results obtained thus far are comparable to those of the haemagglutination-inhibition technique.

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<td>Hypertension and Coronary Heart Disease: Classification and Criteria for Epidemiological Studies First report of the Expert Committee on Cardiovascular Diseases and Hypertension</td>
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<td>169</td>
<td>Joint WHO/FAO Expert Committee on Zoonoses Second report</td>
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<td>170</td>
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<td>171</td>
<td>Mental Health Problems of Aging and the Aged Sixth report of the Expert Committee on Mental Health</td>
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<td>172</td>
<td>Expert Committee on Biological Standardization Twelfth report</td>
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<td>Joint WHO/FAO Expert Committee on Radiochemical Methods of Analysis Report</td>
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<td>174</td>
<td>Expert Committee on Hygiene and Sanitation in Aviation First report</td>
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