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WORLD HEALTH ORGANIZATION
TECHNICAL REPORT SERIES
No. 61

DIPHTHERIA AND PERTUSSIS VACCINATION

Report of a Conference of Heads of Laboratories Producing Diphtheria and Pertussis Vaccines

Dubrovnik, Yugoslavia, 13–18 October 1952

WORLD HEALTH ORGANIZATION
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GENEVA
MAY 1953
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DIPHTHERIA
AND PERTUSSIS VACCINATION

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CONFERENCE OF HEADS OF LABORATORIES PRODUCING
DIPHTHERIA AND PERTUSSIS VACCINES

Dubrovnik, Yugoslavia, 13-18 October 1952

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INTRODUCTION

In January 1949 the WHO Expert Committee on Maternal and Child Health recommended that a technical conference should be convened on procedures for active immunization against common diseases of childhood.

In May 1949 a group of consultants was convened by the Director-General to furnish technical advice regarding this recommendation. In their report it was recommended that a conference on immunization procedures be held, and that its agenda be restricted mainly to techniques of production and evaluation of immunizing agents against diphtheria and whooping cough.

This recommendation was adopted by the Second World Health Assembly.

The Third World Health Assembly decided to call the conference in 1951, but for budgetary reasons the convening of the conference was postponed until 1952.

The conference—held at Dubrovnik, Yugoslavia, 13-18 October 1952—was organized jointly by the WHO Division of Communicable Disease Services, the Division of Therapeutic Substances, and the WHO Regional Office for Europe.

The conference was opened by Dr. N. D. Begg, WHO Regional Director for Europe.

Dr. G. S. Wilson was elected Chairman, Dr. V. Vuksanović, Vice-Chairman, and Dr. P. L. Kendrick and Dr. L. Greenberg, Rapporteurs.

The provisional agenda was adopted.

GENERAL CONSIDERATIONS

It should perhaps be made clear at the start that, by its terms of reference, the conference was restricted to consideration of the preparation and the use of prophylactic agents in the control of diphtheria and whooping cough. This does not mean, however, that the conference in any way...

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\(^a\) *Off. Rec. World Hth Org.* 19, 46
\(^c\) *Off. Rec. World Hth Org.* 21, 182
\(^d\) Resolution WHA3. 71.3.1, *Off. Rec. World Hth Org.* 28, 29
underestimates the part played by hygienic measures in combating these two diseases. Indeed, the greater the activity with which hygienic measures are applied, the greater is the success that can be looked for from the use of prophylactic immunization.

It is between 40 and 45 years since the first attempts were made at active immunization against diphtheria and pertussis. Success at first proved elusive and, although it may now justly be claimed that diphtheria can be controlled by the proper use of modern prophylactics, the disease continues to be a serious problem in many parts of the world. Protection against pertussis has proved even more difficult and it is only within the past decade that effective vaccines have become available. However, since they have not yet been employed on a sufficiently large scale, it has still to be shown that their proper use will, to all intents, eliminate the disease from a country, as has proved possible with diphtheria.

Long experience of immunization against diphtheria has shown that the disease will not be brought under control unless a sufficient proportion of the susceptible population is immunized and kept immune by reinforcing injections. The failure to control the disease in some countries is due to the neglect of these conditions. Success in fulfilling them cannot be achieved without the use of really potent prophylactic agents. The conference has therefore paid particular attention to the preparation of diphtheria prophylactics and to the measurement of their properties against the appropriate biological standard. Only by careful control of the quality of biological products can satisfactory results be hoped for. Many effective preparations are available, but there are several others that are less potent. The conference has given its opinion on the relative efficacy of the different types of prophylactics and has indicated the place of each type in the control of the disease. For the primary immunization of children in mass-immunization campaigns, adsorbed toxoids\(^a\) administered in two doses are undoubtedly the products of choice.

The preparation and biological control of pertussis vaccines are not as well advanced as with diphtheria toxoids. Indeed, the present position is far from satisfactory. Effective vaccines can be made, but it is not possible to define methods of preparation that will ensure consistently good results. Vaccines manufactured by some highly reputable firms have been shown by field trials to be practically devoid of immunizing power. In these circumstances reliable potency-tests, controlled by stable biological standards, are essential. After very careful consideration the conference has reached the conclusion that the only reliable potency-test available at present is to be found in a carefully controlled field trial. It is recognized, however,

\(^a\) In this report, the expression "adsorbed toxoid" is used to designate those preparations which have been variously named "adsorbed" or "precipitated" toxoids.
that certain laboratory tests show considerable promise, particularly the intracerebral mouse-protection test. This test has a large inherent error, but if it is properly carried out it appears to be able to distinguish between vaccines which in field trials show good protection of children and those which show little or no protection. The conference considers that a vaccine giving a poor result in this test should not be issued for use. Although it is not yet known how to ensure reproducible results in the preparation of pertussis vaccine, the conference summarizes the present state of knowledge and indicates those technical procedures which, judged by the results of field trials, appear to be satisfactory, and those which should be avoided.

In recent years combined diphtheria-pertussis vaccines, some with the addition of tetanus toxoid, have been used on an increasing scale. There are a number of scientific and practical problems to be solved before their general use can be recommended. The conference points out that comparatively little information is available on the mutual influence of the individual components on the effectiveness of the vaccine. There is some reason to believe that the result of giving a combined vaccine is determined both by the balance of the different antigens in the mixture and by the degree of latent immunity in the child receiving the injection. Accurate information can be obtained only by careful trials made in the laboratory and in the field. Until these have been carried out, the use of combined vaccines must be considered as necessarily empirical.

It should be noted that the recommendations made by the conference apply to normal children. The existence of medical grounds of contraindications to the immunization of individual children must be left to the decision of the doctor responsible for performing the injections.

This report is divided into three main sections, the first dealing with diphtheria, the second with pertussis, and the third with combined vaccines. The sections on diphtheria and pertussis are each introduced by a historical review. Technical details of vaccine production are included in two annexes.
Part I

DIPHTHERIA

Historical Review of Diphtheria Prophylactics

The etiology of diphtheria, which was at one time termed "croupous" or diphtheritic sore-throat, was elucidated almost simultaneously by Klebs in 1883 and Loeffler in 1884. A few years later Roux & Yersin demonstrated the existence of diphtheria toxin by showing that microbe-free culture filtrates when injected into guinea-pigs gave rise to a pathological picture closely simulating that found in children dying from the disease.

In 1890 von Behring showed that the serum of animals which had received sublethal doses of diphtheria toxin acquired the power of specifically neutralizing the action of toxic filtrates. This important discovery led to the development of antitoxin or serum therapy in diphtheria.

In 1897 Ehrlich developed a method for measuring diphtheria toxin, and his unit for antitoxin has since been adopted as a standard throughout the world.

In 1908 a skin test for estimating the state of immunity of a child, and hence the effectiveness of an immunization procedure, was developed by Schick. Very small amounts of diphtheria toxin were injected intracutaneously; no skin reaction was observed if the child possessed a sufficient amount of circulating antitoxin. However, when this was deficient, a reaction in the form of a red area 1-3 cm in diameter appeared at the site of injection in the course of 24-72 hours. Children may be Schick-tested before and after a course of immunization, and the measure of immunity conferred expressed as the Schick-conversion-rate (SCR).

The abbreviations used in the text have the following meanings:

- ADF: adsorbed dissolved flocules
- APT: alum-precipitated toxoid
- FT: formol-toxoid
- NAFT: natural adjuvant factor toxoid
- PDF: precipitated dissolved flocules
- PT: phosphate toxoid
- PTAH: purified toxoid, aluminium-hydroxide adsorbed
- PTAF: purified toxoid, aluminium-phosphate precipitated
- SCR: Schick-conversion-rate
- TAF: toxoid-antitoxin-flocules
- TAM: toxin-antitoxin mixture (now also used for toxoid-antitoxin mixture)
In early attempts at active immunization against diphtheria an almost neutral mixture of diphtheria toxin and horse antitoxin (TAM) was employed, first by Smith 83, 84 for immunizing horses, and later for children by von Behring, 8 Park, 33, 34 and others. There were a number of technical difficulties involved in the preparation of an effective and safe toxin and antitoxin mixture, and fatal accidents occurred. Nevertheless large numbers of children were inoculated with this preparation, particularly in Germany and in the United States of America.

The disadvantages of TAM were, however, eventually avoided by the discovery of the innocuous but specifically antigenic modification of toxin —toxoid. Formal-toxoid (FT) was independently discovered a number of times, for example by Glenny in 1904, 19 and Ramon in 1923 39, 40 for diphtheria toxin; and Löwenstein in 1904 39 for tetanus toxin. In 1922 Ramon 37 observed that, when unit volumes of diphtheria toxin were mixed with varying amounts of antitoxin of known titre, the first mixture to flocculate was neutral as measured by animal tests. This was rapidly developed as a means for measuring toxin, toxoid, and antitoxin.

Diphtheria FT was extensively exploited for the immunization of horses and children because of its absence of toxicity and consequent safety even when injected in fairly large quantities (Glenny, Hopkins & Pope, 18 Glenny, Hopkins & Pope; 19 Ramon; 39, 40, 41 Park et al. 85).

The immunization course for children receiving FT was three spaced injections. Provided the children received their full immunizing course, a high SCR was obtained and a fall in the incidence of diphtheria occurred (Martin et al. in France; 100 Fitz-Gerald in Canada; 15 Van Boeckel in Belgium; 56 and Sdrodowski in Germany 28).

Unfortunately, the antigenicity of different samples of FT varied considerably; this was not related to their toxoid content. Also, the titres of toxin in those days were very low compared with those obtained in later years. In England particularly, FT was not considered ideal, as the incidence of local reactions was rather high. For some time FT was replaced by washed toxoid-antitoxin-flocules (TAF) which provoked markedly few reactions (Harries; 21 Swyer 85); they suffered, however, from the disadvantage that they contained horse protein, and the immunity conferred was slow in development.

In 1926 Glenny and his colleagues 30 showed that when potassium alum was added to crude FT a precipitate was formed —alum-precipitated toxoid (APT)— and that the precipitate was far more antigenic than the parent toxoid solution. The increase in antigenicity for a single injection was of the order of a hundredfold. The original preparations were unsuited for human prophylaxis owing to the severity of the local reactions they produced. Later, however, Glenny & Barr 17 found that crude APT could be
washed with saline, with the effect that, while retaining its antigenic potency, it gained in purity (Lf/mg N) and lost most of its irritant properties.

Because of the greatly enhanced antigenic potency of APT over its parent FT, the immunization course was reduced to two spaced injections, followed by a reinforcing dose on school entry. The volume of the preparation given was much reduced. There is no doubt that children who receive a full course of injections with good-quality APT are well immunized against the disease.11

APT soon came into use in many other countries but, because little was known of its essential chemistry, a considerable amount of material labelled APT was issued for human prophylaxis which had little relationship with that described by the authors (Bousfield 5, 6, 7). In order to protect the general population against grossly inferior material some licensing authorities imposed new minimal-requirement tests which were far more exacting than any previously described.10

The two main technical disadvantages in the preparation of APT are: (1) it is essentially an empirical procedure, and (2) as a corollary to this, the antigenicity of the final product is not uniform when it is prepared in different laboratories. Moreover, the incidence of local non-specific reactions after injection of APT varies greatly from batch to batch.

In recent years an attempt has been made to avoid these disadvantages, and to determine the optimal composition of an adsorbed prophylactic for the immunization of children.

In brief, it was found that the mineral component of APT is a mixture of aluminium hydroxide and phosphate. The hydroxide is by far the more powerful adsorbing component for toxoid as well as for other nitrogenous material. The phosphate is much weaker in this respect but more selective. It was also found that when purified toxoid was added to a suspension of preformed aluminium-phosphate gel, it was efficiently adsorbed at pH 5 but not at pH 7.4, although if it was held at pH 5 for some weeks it was very incompletely eluted on adjustment to pH 7.4. Such a matured preparation proved to be highly antigenic in animals. The antigenicity (antitoxin-producing power) of this prophylactic, termed purified toxoid, aluminium-phosphate precipitated (PTAP), increased with an increase in the amount of aluminium-phosphate gel injected, reaching a maximum at 3 mg for guinea-pigs and 5-7.5 mg in young children.

With 5 mg of aluminium phosphate (AlPO₄) and 5 LF or more of purified toxoid, an SCR of over 95% was obtained in four weeks after a single injection; 15 months later 90% of the children were still Schick-negative (Holt & Bousfield 23). Reversing the procedure, namely using a constant amount of aluminium phosphate and varying the amount of toxoid adsorbed, it was found that there was a fairly well-defined critical amount of
toxoid required. Amounts greater than 5 Lf resulted in only a small increase in SCR, whereas amounts less than this resulted in a rapidly decreasing SCR (Bousfield & Holt 9).

A point of some considerable immunological significance is that a single injection of PTAP was found to induce in guinea-pigs some five to six times as much antitoxin as the best-quality APT administered in the same volume, and that the scatter of values was much less with PTAP (Barr & Llewellyn-Jones 1). The difference between these two prophylactics was, however, very greatly reduced when two spaced inoculations were used.

A similar diphtheria prophylactic has been developed using preformed aluminium-hydroxide gel as the mineral carrier (Schmidt & Hansen; 51 Scheibel 45, 46). The amount of hydroxide used is approximately 2.9 mg of aluminium hydroxide per child-dose, with 50 Lf of purified toxoid. This prophylactic has proved to be very effective when two or three spaced inoculations are employed.

Fundamental Considerations

Mechanism of immunity in diphtheria

Though the possibility that antibacterial substances might play a part in protection against diphtheria has been envisaged from time to time, the opinion has almost universally been held that antitoxin alone is responsible for immunity to this disease. Of recent years, however, consideration has again been given to the possible role of antibacterial immunity, mainly as the result of the frequent failure of antitoxin to check the progress of the disease in patients suffering from infection with the more virulent strains of diphtheria bacilli. This failure may have been due to the presence in the infecting bacillus of a toxic element that was absent from the strains used for preparing the antitoxin, or to a difference in the invasive power of the organism, or to a modification in the properties of the therapeutic antiserum as a result of the more modern methods of purification, or to some other cause.

Up to the present, attempts to immunize against diphtheria have been based mainly on the assumption that an antitoxic immunity is alone of importance. It must be recognized, however, that the methods of preparation of diphtheria prophylactics in the past have not excluded the presence of a certain amount of bacterial protein which may have given rise to some degree of antibacterial immunity. Experiments on guinea-pigs have shown that a pure antibacterial serum may protect against infection with a small number of diphtheria bacilli, but there is no doubt that only an antitoxic serum can be relied upon to neutralize the specific diphtheritic toxin.
It may be concluded that antitoxic immunity is of major, if not of supreme, importance in protection against the disease, but it is at present difficult to exclude the possibility that antibacterial immunity may play some part, particularly in defence against severe infections.

The Schick-test

The Schick-test is universally used for the determination of susceptibility to diphtheria toxin. In order that the results obtained and reported in different countries should be comparable, the conference recommends that the reagents used in the test should be standardized. This should be done preferably in the way laid down in the British Therapeutic Substances Act, 1925, though it is recognized that alternative methods may yield satisfactory results. The WHO Expert Committee on Biological Standardization is therefore requested to consider the provision of a reference standard Schick toxin, and the adoption of the regulations laid down in the British Therapeutic Substances Act, governing the standardization of Schick toxin. These are incorporated in Annex 1 (page 44).

The conference points out that a Schick-test can act as a secondary stimulus to antibody formation in an immunized child. Therefore it is recommended that, as far as possible, field trials of diphtheria prophylactics should be carried out by methods which avoid the possibility of this effect confusing the immunological results.

Requirements of effective prophylactics

Before the relative merits of the various diphtheria prophylactics can be assessed, it is necessary to define the criteria by which they are judged. The conference considers that an effective prophylactic should, as nearly as possible, meet the following requirements:

1. safety, including not only bacterial sterility and the absence of free toxin, but also freedom from the possible risk of activating latent infections or giving rise to sensitization or to other such disturbances;
2. innocuity, meaning the freedom from the risk of giving rise to severe local or general reactions;
3. ability to stimulate a satisfactory degree of immunity of sufficiently long duration;
4. ability to produce immunity in a small dose, contained preferably in a volume of 0.5-1.0 ml, when given twice at an interval of one month;
5. stability for at least one year under ordinary conditions likely to be met with in practice;
6. capability of being prepared on a mass-production basis, with easily obtained reagents, and with uniformly reproducible results.
Current prophylactics

The various prophylactics which have been used in the past have been discussed in the historical review. The conference lists the following diphtheria prophylactics as those acceptable for use at the present time:

(1) FT (formol-toxoids—crude and purified);
(2) TAF (toxoid-antitoxin-flocules);
(3) APT (alum-precipitated toxoid);
(4) PTAP (purified toxoid, aluminium-phosphate precipitated);
(5) PTAH (purified toxoid, aluminium-hydroxide adsorbed).

Newer products on which further information is needed are:
(a) NAFT (natural adjuvant factor toxoid);
(b) protamine toxoid;
(c) Mason’s PDF (dissolved flocule solution precipitated with alum$^*$);
(d) Mason’s ADF (dissolved flocule solution adsorbed on to aluminium phosphate).

Choice of prophylactic

Most immunization campaigns against diphtheria are conducted on a large scale, and an attempt is usually made to give either two or three doses of prophylactic during the first immunizing course. Inevitably, however, a certain proportion of the children who receive their first dose are unable, for one reason or another, to complete their course of injections. It is therefore wise to use a prophylactic which is capable of giving rise to a high degree of immunity after even one dose. Nevertheless, the conference is unanimously of the opinion that a single-dose method of immunization cannot, and should not, be relied upon to produce immunity.

Modern adsorbed prophylactics given in two doses at an interval of one month are highly effective and produce an SCR of over 99%. There is little difference between the different products if the full course is given. On the other hand, as already pointed out in the historical review, the response to a single dose of PTAP appears to be rather better than that following APT.

$^*$ It may be noted that throughout this report the word "alum" is frequently used in a loose and strictly impermissible way. It would usually be more correct to replace it by the term "an aluminium derivative of", implying that the product referred to is not necessarily prepared from potassium alum. To avoid such a cumbersome expression, however, and for reasons of general convenience and usage, the word "alum" is adhered to. A note on the chemical complexity of aluminium precipitates is included in Annex I (page 44).
FT in three doses is effective, giving an SCR of 95% to 97%, but these figures are a little lower than those attainable by the use of adsorbed products. It is clear that good results may be obtained when either fluid or adsorbed toxoids are used. However, in view of the desirability of protecting in mass campaigns a high proportion of children with as few doses as possible, the conference recommends that an adsorbed product administered in two doses should be used for the primary immunization of young children.

Recently a purified form of FT has been introduced. In the hands of some workers such a product has given an SCR of 97.7% after two injections at an interval of one month. In other hands purified FT’s prepared by slightly different methods have been found to be less effective, and it is clear that further work is required before the value of these products in routine antipertussis immunization can be assessed.

Methods of use of prophylactics

It is recommended that the dose of adsorbed preparations for a child should be contained in a small volume, preferably in not less than 0.5 ml or more than 1.0 ml. Either the subcutaneous or intramuscular route of injection may be employed. The dose interval should be not less than 4 weeks nor more than 12 weeks.

For children under ten years, it is considered that a Schick-test is unnecessary, and that the reinforcing dose should be contained in the same volume as that used for the last of the primary immunizing injections. For children over ten years of age a Schick-test, properly controlled, may be used at the discretion of the physician, more for deciding whether the child is sensitive to the control injection than for finding out whether the child is Schick-positive. When a reinforcing dose is necessary, it may be equal to or smaller than the primary dose.

The first reinforcing dose should be given within five years of primary immunization. If possible a second dose should be given within the following ten years. It is considered desirable to give reinforcing doses to children up to at least ten years of age whether they are Schick-positive or Schick-negative, on the grounds that a Schick-negative child may revert to the Schick-positive state while he is still in danger of contracting diphtheria, and that a reinforcing dose will tend to prevent this from occurring.

For the primary immunization of adults, either adsorbed preparations, TAF, or purified FT may be used. These should be given in smaller doses than those normally used for children, and should be restricted to persons reacting positively to the Schick-test. Schick-positive adults giving in addition a reaction to the control injection, or having a history of diphtheria, should be immunized only with great caution.
Immunization during early infancy

Hitherto, in most countries, diphtheria immunization has been offered to infants towards the end of their first year of life. More recently, because of the demand for combined diphtheria and pertussis immunization, consideration has been given to immunizing infants at an earlier age. Since about 70% of the deaths from pertussis occur during the first year of life, it has been thought advisable by some paediatricians to start immunization against pertussis when the child is 3 months old (see page 41). Insufficient information has so far accumulated to show whether immunization against diphtheria can be satisfactorily accomplished at this early age. Various investigations have shown that the presence in the infant's blood of antitoxin derived from the mother may interfere to some extent with the normal antibody response. It would appear that, under these conditions, antibody is formed by the infant more slowly than if no antibody is present to start with; but there is some evidence—as yet unconfirmed—to suggest that, even though antibody production may be delayed, the final amount of antitoxin produced is very much the same as in infants with no initial antitoxin. Little information, however, is available to show how long immunity produced in this way will last. It is clearly desirable to ensure a degree of immunity that will carry the child through till he is five years or so of age, when he will normally receive a reinforcing dose. If it should be found that immunity produced in early infancy is of less than this duration, administrative considerations will probably render it desirable to keep to the present practice of immunizing infants towards the end of their first year of life.

Control of diphtheria

In the control of diphtheria, attention should be paid to the ordinary hygienic measures recommended for the prevention of spread of infection. Practised alone they will not eliminate the disease, but in conjunction with a well-designed immunization programme they will accelerate the beneficial results obtained by the production of specific immunity.

Further, it should be realized that the mere immunization of a proportion of the population may not suffice to stem the disease in the community. Observations in two or three different countries indicate the necessity of producing a group as well as an individual immunity. In other words, such a high proportion of the population must be rendered immune as to make the continued passage of the diphtheria bacillus from one susceptible person to another very difficult. To obtain this result the exact proportion of children to be immunized will vary from country to country, depending to a considerable extent on the general hygienic conditions. In the USA
and in Great Britain, it would appear that at least 70% of the child population must be rendered and kept immune if the disease is to be brought under control. Otherwise, cases will continue to be notified and, as experience has shown, will occur in immunized as well as in unimmunized subjects—though deaths, it may be added, among the immunized are likely to be rare.

In planning an immunization campaign, arrangements must therefore be made to give primary and reinforcing injections to as high a proportion of children as possible, remembering that unless at least 70% of the child population is rendered and kept immune, the campaign may prove to be a failure, and may bring discredit on the whole method of immunization.

It may be pointed out that, in the control of outbreaks of diphtheria occurring in schools and institutions where a substantial proportion of the children have never been immunized, combined active and passive immunization, especially if accompanied by the temporary segregation of carriers of the diphtheria bacillus, may play a useful part (Fulton et al. 19).

Reactions

The conference recognizes the difficulty of comparing the reactions produced by different prophylactics without defining what constitutes a local and what a general reaction. Any attempt at definition is rendered difficult by the circumstance that in both types of reaction there is a considerable subjective element. Such features as pain, tenderness, malaise, and headache are incapable of exact measurement, and it is therefore felt that an objective test alone can be accepted, even though it fails to take account of all the characteristics of the reaction.

Therefore the conference recommends that a local reaction should be defined as an area of redness of at least 20 mm in diameter present 48 hours after inoculation. A general reaction should be defined as one characterized by the presence of a temperature of 38°C (100.4°F) or higher at any time between 24 and 72 hours after inoculation.

All diphtheria prophylactics in current use will cause reactions to a varying degree. In children under ten years of age, these reactions occur seldom and are generally insignificant. The incidence is probably lower after the use of modern preparations such as PTAP and PTAH than it is after FT and APT, but the difference is small. In older children and adults reactions are fairly common after all these preparations, and may be severe after APT and FT prepared from crude toxoid. TAF, which must be administered in three doses, gives fewer reactions than the other preparations in adults, but it has, of course, the disadvantage that it contains horse protein. Reactions after PTAP and PTAH can be reduced considerably by diminishing the dose of toxoid administered. This may be done
without seriously affecting the immunizing power of these preparations, so long as the amount of carrier used is not correspondingly reduced.

The nature of the substances responsible for giving rise to reactions has not yet been fully determined. It is certain that any impurities in the form of protein in the preparation may cause reactions. *Corynebacterium diphtheriae* in culture produces both specific and non-specific proteins, the former predominating during the first 4-5 days of cultivation, the latter increasing on further incubation. The iron content of the medium is known to influence the proportion of specific to non-specific protein. If this is reduced below the level for optimum toxin production, then the non-specific proteins are reduced to a greater extent than are the specific. There is reason to believe that both the specific and the non-specific proteins are capable of evoking a reaction, although evidence so far obtained suggests that sensitivity is far commoner towards the non-specific than towards the specific protein. Even highly purified toxoids, however, cannot be expected to be entirely non-irritating. Attempts to eliminate the non-specific protein without reducing the potency of the toxoid have so far been only partly successful, and further work on this problem is required.

*Laboratory control of diphtheria prophylactics*

*Antigenicity test.* The antigenicity test recommended by the conference is a minimum-requirement potency test. The test animal is the guinea-pig. A one-dose method of immunizing the guinea-pig is used, and the dose administered is a fraction of the first child-dose. A two-dose method of immunizing guinea-pigs is not used, because such a method makes it easier for a poor-quality prophylactic to pass the test. Though it is appreciated that many different methods can be used for estimating the induced immunity of the animal, a challenge-dose test is adopted for the purpose of simplicity, the challenge dose being expressed in terms of median lethal doses (LD₅₀) of toxin.

(a) *Alum-precipitated and adsorbed diphtheria toxoids*: The test recommended is dependent on the availability of a reference-standard alum preparation. Such a standard aluminium-hydroxide adsorbed toxoid is being considered by the WHO Expert Committee on Biological Standardization. It is hoped that this standard will be adopted and will be available for general use within a year or two. In the meantime, the present International Standard for plain diphtheria toxoid, although not as satisfactory as an adsorbed standard, may be used as the reference preparation. For this purpose it should be employed according to the dosage recommended in the minimum-requirement antigenicity test for formol-toxoids.

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At least 15 normal guinea-pigs weighing between 250 g and 350 g are injected subcutaneously in the wall of the abdomen with 0.5 ml of physiological saline containing 1/25th of the first dose recommended by the manufacturer for injection into children. Between 28 and 31 days later the animals are challenged with 20 LD₉₀ of a suitable diphtheria toxin which, for the purposes of this test, should be one containing at least 20 LD₉₀ per Lf. The test animals are observed for 5 days after the challenge dose, and must show a survival-rate not less than that obtained with the reference-standard toxoid which has been tested simultaneously. The control guinea-pigs are immunized with an amount of the reference-standard toxoid to be specified by the Expert Committee on Biological Standardization after the International Standard for diphtheria toxoid, adsorbed, has been established.

The test will be most satisfactory if the control guinea-pigs have approximately a 50% ± 25% survival-rate. Some laboratories may have to adjust their immunizing doses of both reference and test toxoids to keep within this range.

(b) *Formol-toxoid (FT)*: A similar procedure is adopted for testing FT. However, a less stringent test is needed, because FT is recognized as less potent and has to be administered to man in at least three properly spaced doses.

The procedures to be followed are identical with those described for alum preparations, except that the guinea-pig immunizing dose for the toxoid under test is one-half the first child-dose, contained in 0.5 ml of physiological saline. Further, the reference preparation for formol toxoids is the International Standard for plain diphtheria toxoid. The immunizing dose of the standard for the test animal is 0.5 mg contained in 0.5 ml of physiological saline. After challenge with 20 LD₉₀, 50% ± 25% of the control guinea-pigs should survive. Laboratories not obtaining this result should adjust their immunizing doses accordingly. At the completion of the test the toxoid under consideration should yield a survival-rate at least equal to that obtained with the reference standard.

*Innocuity tests.* (a) *Dermal toxicity test for formol-toxoids only—crude and purified:* Either white rabbits, or white guinea-pigs weighing approximately 300 g, are injected intracutaneously in the back with 0.2 ml of the toxoid containing not less than 30 Lf per ml. Toxic reactions must be absent during the observation period of 48 hours after injection.

(b) *Delayed toxicity—formol-toxoid and adsorbed toxoids:* At least five guinea-pigs are injected parenterally with 5 ml* to the toxoid under test.

* For PTAP preparations the test dose should be at least three first child-doses.
The animals must not show significant symptoms, paralysis, or death during an observation period of 6 weeks or longer.

_Stability_

The conference does not feel in a position to recommend specific thermo-stability tests. It does recommend, however, that whenever possible diphtheria prophylactic should be stored at 2°-4°C. Suggestions by Dr. Holt were noted that satisfactory prophylactics should still pass all the tests under the minimum requirements described earlier (see page 17) after exposure to a temperature of 37°C for 3 months. Observations on accelerated methods of testing the stability of toxoids in the laboratory are very desirable.

_The value of field trials_

The conference is of the opinion that, although the guinea-pig test affords a good index of potency, the final criterion by which a prophylactic is assessed should be its activity in the human subject as judged by Schick-conversion-rates, antitoxin production, and protection against diphtheria.

_Observations on production methods_

In view of the large variety of diphtheria prophylactics in use today, the conference limits its observations on production methods to the absorbed preparations which are regarded as being of most practical value. The manufacturing procedures for some of these preparations are described in detail in Annex 1 (page 44). The conference points out that their manufacture requires considerable practical knowledge and experience, without which success is not likely to be attained.

_Further research_

The conference notes that research is being carried out on the use of oral and percutaneous immunization for the reinforcement of immunity against diphtheria.

It also notes and expresses the need for continuing studies on infant immunization. Recent observations have shown that infants immunized at 3-7 months of age may respond well to a prophylactic combining diphtheria toxoid, tetanus toxoid, and pertussis vaccine. Information concerning the duration of immunity in infants when they are immunized with diphtheria prophylactic at this early age is lacking, and it is very desirable to learn whether this immunity will last until the child reaches school age—namely, for a period of four to six years.
Further studies on adverse reactions occurring with the different prophylactics in both infants and adults are also needed. It is understood that work is in progress designed to reduce the irritating property of even the present purified product without diminishing its antigenic potency.

There is still too little information available on the stability of toxoids, particularly those to be used in tropical countries. It is recommended that observations should be made on the potency of such preparations after storage at high atmospheric temperature for periods of up to two or three years.

The ideal diphtheria prophylactic capable of giving rise to a high degree of immunity at all ages without causing either local or general reactions still remains to be found.

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Part II

PERTUSSIS

Historical Review of Immunization against Whooping Cough

In 1906 Bordet & Gengou\textsuperscript{4} reported the isolation of the causative organism of whooping cough. During the next few years, vaccines of the Bordet-Gengou bacillus, prepared and administered in various ways, came to be used fairly widely in different countries, on the basis more of an assumption than proof of their value. A few reports appeared in the literature but, in general, precise information on methods of preparation, testing, and use of these vaccines was lacking. The numbers of children were small and there were no control groups, which made the assessment of the results very difficult.

In 1931, attention was directed by Leslie & Gardner\textsuperscript{22} to different antigenic phases of \textit{Haemophilus pertussis}—a phenomenon now generally interpreted in terms of smooth to rough variation rather than in terms of four stable phases. Their work was important because it emphasized the necessity for selecting smooth antigenic strains for vaccine preparation.

\textit{Review of field trials}

There is space here to review only a few of the more important field trials that have been carried out. The first studies to show real evidence of protection were those of Madsen.\textsuperscript{23, 26} In connexion with these, use was made of the cough-plate diagnostic method of Chievitz & Meyer,\textsuperscript{9} enabling freshly isolated organisms to be used in the preparation of the vaccine. Madsen’s experience was of particular interest because of the occurrence of two outbreaks in the Faeroe Islands, one in 1924 and one in 1929. The first outbreak had started before the vaccine injections were begun, and most of the children in both the vaccinated and the non-vaccinated group contracted whooping cough. However, only 5 deaths occurred among the 2,094 vaccinated children in comparison with 18 among 627 non-vaccinated, or case-fatality rates of 0.24\% and 2.9\% respectively. In the second outbreak, the vaccine injections were completed shortly beforehand, and some evidence of prophylaxis was found in the fact that only 75\% of 1,832 injected children contracted whooping cough, compared with 98\% of 446 non-injected controls. One fatal case occurred in the vaccinated group in comparison with eight among the controls.
In 1933 Sauer,\(^{30}\) in the USA, reported on his use of pertussis vaccine in 300 young non-immune children between 1928 and 1933, and concluded that a good degree of immunity was produced. Sauer, in this and subsequent studies, emphasized the use of recently isolated, strongly haemolytic strains for vaccine preparation, and the importance of scraping the growth from the medium rather than washing it off. Sauer estimated his total dosage to be 70,000 to 80,000 million in comparison with Madsen's estimated 22,000 million. The main difference between Sauer's vaccine and Madsen's appears to have been the use of human instead of horse blood in the Bordet-Gengou medium, and the use of a larger dosage for injection. Substitution of human blood was based on convenience; it was also thought to avoid any possibility of sensitization due to the carrying over of horse protein to the vaccine from the medium.

In the Michigan Department of Health Branch Laboratory in Grand Rapids, Michigan, after the establishment of a diagnostic cough-plate service, a study on active immunization against whooping cough was initiated by Kendrick & Eldering in 1934. Emphasis was placed upon the inclusion of a pre-designated control group for each vaccinated group under study. The work developed into a series of studies, each with its particular objective. In the first trial, reported in 1939 and 1940 (Kendrick & Eldering;\(^{18}\) Kendrick\(^{16}\)), the one question to be answered was: "Is there a protective response to the pertussis vaccine under trial?" In the second (Kendrick\(^{18}\)), an alum-precipitated vaccine was used; and in the third (Kendrick\(^{16}\)) pertussis vaccine was combined with diphtheria toxoid and was alum-precipitated. Subsequent series were not strictly controlled, but directed rather towards incorporation of an immunization programme into the local health department.

The total dosage in the first trial was estimated to be 70,000 million organisms, given in four injections, at weekly intervals, of 1, 1.5, 1.5, and 3 ml respectively, one-half of the last dose being injected in each arm. In the second trial three injections were given of 1 ml each of a 10,000 million per ml suspension of plain vaccine in one group and alum-precipitated in another, with an interval of one week between the first and second, and four weeks between the second and third injections. In the third trial the first injection was of plain vaccine, and the second and third were combined pertussis vaccine and diphtheria toxoid alum-precipitated; otherwise the vaccine was given as in the second trial.

In the first trial there were 4,212 children between the ages of 8 months and five years—1,815 in the vaccinated group and 2,397 in the control group. The past histories of the two groups were carefully compared, and a note made not only of the previous occurrence of whooping cough but also of the incidence of other childhood diseases. They were found to be
similar, and suitable for comparative analysis. On the basis of person-years observation, the annual attack-rates per 100 for the vaccinated and control groups were 2.3 and 15.1 respectively. Also, the 52 attacks that did occur in the vaccinated group were rated as milder than the 348 attacks in the unvaccinated group. There were no deaths in either group. Subsequent trials, reported and unreported, confirmed the findings of the first, as did a statistical approach devised by Sargent & Merrill for the comparison of reported cases in the whole community and in a vaccinated group. This latter method was applied by Weiss & Kendrick to a sample of the vaccinated group in the field trial and to the community of Grand Rapids, with the result that the protection-rate obtained was practically the same as in the field trial. Furthermore, a vaccine prepared in much the same way by the Michigan Department of Health Laboratories gave similar results in a controlled field study in Binghampton, New York State, by Perkins and his associates. Kendrick also reported favourable results when pertussis vaccine was alum-precipitated, and when it was combined with diphtheria toxoid.

An alum-precipitated vaccine prepared from organisms grown on Bordet-Gengou medium was tested by Bell in a carefully controlled trial among children of the general population of Norfolk, Virginia. He used an estimated total dosage of 20,000 million organisms, in two injections, with a 4-week interval between injections. He reported in 1941 that substantial protection had been conferred by the vaccine. In a later trial he used an alum-precipitated mixture of pertussis vaccine combined with diphtheria toxoid, and again obtained evidence of substantial protection.

It must be pointed out that not all workers have obtained equally promising results. Essentially negative findings in a controlled field trial in Cleveland, Ohio, were reported by Doull and his associates in 1939. The only obvious differences between the vaccine under test in this study and that, for example, employed in Grand Rapids were the use of human rather than sheep blood in the medium for production, and the washing of the organisms with distilled water before resuspension in phenolized salt solution (for how long is not indicated). It is pointed out that at that time no laboratory protection-test was available as a basis for comparison.

Publications on the use of pertussis vaccine in Great Britain are of unusual interest and relevancy, because in controlled field trials they failed to demonstrate protection in some instances and demonstrated varying degrees of protection in others, depending on the vaccines under test. Reports up to 1933 were summarized in an editorial in the British Medical Journal.

In 1945 McFarlan and his associates reported the results of controlled field trials carried out during 1942 for the Medical Research Council
(MRC) of Great Britain. They observed no significant difference in the incidence or severity of the disease between vaccinated and unvaccinated groups. On the assumption that the antigens used in the successful and unsuccessful trials must have differed in some material though unknown way, it was decided by the MRC Whooping Cough Immunization Committee to carry out new trials and, for comparison, to use vaccine of American origin which had been reported to be effective in controlled studies. In the Committee's report of 1951,7 the methods used for controlling the field trials are described, including the use of injections of an anticitarrhal vaccine in the non-vaccinated control group. Vaccines prepared in several different laboratories were selected for study. A total of 7,558 children distributed in different areas were kept under observation; approximately half of them acted as controls. The attack-rates among the children with "home exposures" and with "other exposures" were calculated for the several vaccinated groups, and considerable differences in the degree of protection were observed. For example, among "home exposures" the attack-rates among the vaccinated children ranged from 7.3% with one vaccine to 30.4% with another; the attack-rates in the corresponding unvaccinated groups were 79.5% and 90.5%. The observers considered that the attacks which did occur among vaccinated children were, on the average, less severe and of shorter duration than in the unvaccinated.

Lack of space requires the omission of other trials that have contributed to our present experience of active immunization. Those briefly sketched serve to illustrate both successful and unsuccessful attempts at active immunization and to emphasize the great difficulties inherent in mass studies. They also show the need for establishing criteria by which to measure the product under study as well as the methods of testing in the field.

Many studies have been carried out during the past few years in the development of methods for testing the potency of pertussis vaccines in the laboratory. In Australia, mouse-protection tests using the intranasal route of infection have been studied, whereas in the USA the intracerebral route for administering the challenge dose has been mainly used.

In the field trials in Grand Rapids, Michigan, it was recognized early that progress in active-immunization studies must remain almost at a standstill until some means could be found for defining the antigenicity of a particular batch of pertussis vaccine. Without this it was impossible to decide whether, in two field trials, the vaccines behaved alike or not. Moreover, workers were held back from attempts to improve vaccines because there was no practical way of measuring the potency of their experimental products. During the period 1943-6, a study was made of various methods of testing pertussis antigens by the mouse-protection test. The procedure that seemed to offer the greatest promise as a test for potency
was one in which intracerebral challenge was used, as reported in 1947 by Kendrick, Eldering, and their co-workers.\textsuperscript{19}

Considerable experience of the intracerebral mouse-test has now accumulated, in different laboratories. Since 1948, after a trial period of approximately two years, all pertussis vaccines released for distribution by manufacturing laboratories in the USA have been required by the National Institutes of Health (NIH), Bethesda, Md., to pass such a test.\textsuperscript{20} In experimental work, it has served to give workers a basis for judging progress, and in many laboratories a good mouse-protection test is considered the best single criterion for selection of a culture for vaccine production. The value of this test is now being studied in the British field trials.

**Fundamental Considerations**

*Mechanism of immunity in pertussis*

The essential antigen responsible for the stimulation of immunity in pertussis has not yet been identified. The pertussis organism produces a toxin in addition to its somatic and capsular antigens. Theoretically, immunity could be stimulated by either the toxin or the bacterial antigens. All the work to date has failed to demonstrate the value of antitoxic immunity. Not only can no antitoxin be demonstrated in the blood of patients convalescent from whooping cough, but vaccines containing no toxin have yielded excellent results in field trials. It is very doubtful, therefore, whether the toxin plays any significant role in stimulating an immune response to the pertussis bacillus.

The immune response of the vaccinated child has been studied in terms of agglutinins, complement-fixing antibody, opsonocytaphagic antibody, and skin reactivity. Several workers have found a fairly close association between agglutinins and protection in field trials, but it is still impossible to say whether the agglutininogen is or is not the antigen responsible for immunity. The few workers who have used the opsonocytaphagic test have obtained results in general agreement with those of the agglutination test. The number of studies so far reported on the complement-fixation test is too small to justify any statement on the relation between the results of this test and the degree of protection conferred by the vaccine. Most of the recent studies of the skin-test reaction have been done with Flosdorf’s\textsuperscript{12} agglutinogen. The experience with this test in the hands of different workers has been variable. It is clear that further work is needed before these various tests can be evaluated as a means of measuring the child’s immune response, or as a method for assessing the antigenic potency of vaccines.
Requirements of satisfactory vaccines

These are essentially the same as those already set out under diphtheria (see page 12), with the exception that under (i) the proviso about the absence of free diphtheria toxin does not apply, and it is as yet impossible to produce a satisfactory immunity in two doses (4).

Current prophylactics

For convenience, the current vaccines are divided into two groups, those grown on a solid medium and those grown on a fluid medium. As a rule the solid media are of the Bordet-Gengou type, varying mainly in the type of blood used. A few laboratories use a blood-free solid medium of the casamino acid type containing charcoal. The fluid media employed are of the casamino acid type with various modifications. The vaccines prepared in these media are used either as whole suspensions or as suspensions of the organisms after these have been removed from the medium and resuspended. In some instances the vaccine is a plain suspension; in others it is prepared as an adsorbed product. Studies are in progress with several types of soluble antigens, but no information on these is yet available.

The vaccine suspensions may or may not have been subjected to some degree of washing and may have been killed by various methods mostly making use of chemical agents.

Methods of use

The dosage of pertussis vaccine employed by different workers has been extremely variable. It is difficult to make an exact measure of the degree of variability because the methods used by different manufacturers for the estimation of the bacterial content of vaccines vary widely. For example, a vaccine standardized by one method may give a result in terms of numbers of bacteria three or four times that of the same vaccine standardized by another method. Of the three methods in current use for the standardization of vaccines—namely, bacterial counts, micro-Kjeldahl nitrogen content, and opacity—the conference considers that the last, though far from ideal, offers the best hope of obtaining uniform results.

If opacity is to be used for this purpose, a reference opacity standard will be required. There is at present in use in the USA a standard employed by the NIH. The turbidity of this standard is believed to correspond approximately to a count of 10,000 million organisms per ml. This turbidity is given the arbitrary figure of 10 opacity units. It is proposed in future to indicate the strength of pertussis vaccines in the USA in terms of those units rather than of numbers of bacteria. The WHO Expert Committee on
Biological Standardization has authorized the establishment of this standard as the International Reference Preparation for Opacity.

The results of field work and other observations indicate that either plain or adsorbed pertussis vaccines may be used for immunizing children over six months of age, although adsorbed vaccines have not been shown to give superior results and the incidence of reactions is higher. The conference recommends that primary immunization should be started between the 3rd and 12th months of life (see also page 41), and that the total dosage should represent approximately 60 opacity units, given in three injections at monthly intervals, each dose in a volume of 0.5 to 1.0 ml. A reinforcing dose of the same size as the last of the primary series is recommended at two to four years of age. There is some evidence that the very young infant responds better to an adsorbed vaccine given in three injections, followed by a reinforcing dose before the age of 18 months.

It is pointed out that bacterial content per se is not the only factor in determining the protective power of a vaccine. In various field trials vaccines with a high bacterial content have given results inferior to others of different manufacture containing half the number of organisms. In order to minimize reactions, the minimum number of organisms consistent with the production of a satisfactory degree of immunity should be given.

Field-trial results

The more important field trials on pertussis vaccine have been referred to in the historical review (page 22).

It is regrettable that no carefully controlled field trials have as yet been completed with vaccine prepared in a fluid medium. Vaccines in all successful field trials to date have been prepared on Bordet-Gengou medium, and dispensed in either the plain or the adsorbed state. Current trials with a fluid-medium vaccine suggest that protection may be as good, but the results are incomplete.

The latest reported field trials of the MRC investigation indicate the measure of protection that can be obtained by the use of a vaccine on Bordet-Gengou medium. After exposures in the home, 80%-90% of the unvaccinated children suffered from attacks of pertussis, whereas children vaccinated with the best vaccine showed attack-rates of only about 8%. Other vaccines gave rates ranging from 22% to 30%.

Design of field trials

It is impossible to place too much emphasis on the proper design of a field trial. A poorly designed trial is not only useless but may be mis-
leading. In the MRC trials, the following guiding principles were put into practice:

"(a) Before the substance under test is used on a large scale in the field it must have satisfied accepted criteria for the absence of toxicity, severe reactions, and untoward sequelae, and must have been administered to a number of volunteers without ill effect. There must also be presumptive evidence from animal or volunteer experiments that the prophylactic is likely to be of real value.

"(b) Before the main investigation is planned, a small-scale preliminary trial should, if possible, be undertaken; a 'pilot' field trial quickly reveals unforeseen difficulties, and often indicates how these may be overcome.

"(c) For the main study two similar groups of subjects are required, one to act as a test group and the other as a control group. The two groups must be chosen so that when considered as groups (and not as individuals within groups) they are alike in all relevant respects, except that one group has been treated and the other has not.

"(d) Both groups must be observed over the same period of time and with the same care and intensity. The observers should not know which of the subjects are in the test group and which in the control group; the subjects—or their parents—likewise must not know whether they are members of the test or control group.

"(e) As far as possible the diagnosis of the disease being studied must be confirmed by objective criteria.

"(f) All observers should be aware of the whole plan of the investigation and the nature of the substances being administered. The subjects—or their parents—must, as far as is possible, be given full information about the aims of the investigation and about the presumed effects of the substances to be administered.

"(g) Investigations should, if possible, be carried out in a number of widely separated areas simultaneously; in all areas the organization and day-to-day work must conform to a central plan.

"(h) The trials should be terminated as soon as satisfactory statistical evidence has been obtained."

Valuable information on the design and execution of field trials will be found in the article by Bell on combined immunization against diphtheria and whooping cough.

In view of the difficulty of organizing such trials and of finding field workers with the necessary training and experience, the conference is of the opinion that the continuation of the MRC's field trials is highly desirable, and points out that the results obtained are of considerable value to other countries.

Reactions

Reactions after the injection of pertussis vaccines are admitted to be more frequent than those after the injection of diphtheria prophylactics made with purified toxoid. They are seldom, however, sufficiently serious to prevent completion of the course of immunization. The relation between the size of dose and the severity of the resulting reaction has not been
established with certainty, but excessive dosage should undoubtedly be avoided. There is considerable evidence that too superficial an injection of vaccine is likely to provoke local reactions.

Sterile abscesses, which are rarely, if ever, observed after the proper administration of plain vaccine, are noted more frequently after the injection of adsorbed products, particularly when these are administered subcutaneously. The intramuscular injection of either plain or adsorbed vaccine apparently minimizes the incidence and severity of local reactions.

Systemic reactions, as evidenced by increase in body temperature, are fairly common during the first 24 hours and may extend longer. They are not, however, considered to be a major concern.

Encephalopathy is a rare complication during the course of whooping cough, and has been reported to occur occasionally after vaccination with pertussis vaccines. There is no conclusive evidence that this complication is directly attributable to the vaccine administered. Some of the cases of post-vaccinal encephalopathy that have occurred have been encountered in children with a personal or family history of epilepsy or other neurological disorders. They would not be expected to occur in normal healthy children.

The possible relation between pertussis-vaccine injections and paralytic poliomyelitis is discussed in Part III (see page 38).

The conference agreed that the criteria for indicating the degree of local and systemic reactions should be the same as those outlined for diphtheria toxoid.

Control procedures

The need for a specific laboratory test which could be used for estimating the protective potency of a vaccine in place of evidence provided by field trials was clearly recognized by the conference. The mouse-protection potency test using intracerebral challenge has been used extensively and it was agreed that, although this did not permit an exact estimation of the protective potency of a given vaccine, it was able apparently to distinguish between vaccines of comparatively high and comparatively low activity. The inaccuracies of the method resulting primarily from variations in sampling that are inherent in a binomial distribution are such that a high degree of precision can be attained only by the use of an inordinately large number of mice. The accuracy of the potency estimate increases roughly in direct proportion to the square root of the number of animals employed and to the slope of the dosage-response curve, which is characteristically low in this type of measurement. It is considered, however, that even though these statistical limitations prevent an exact estimation of the potency of a
given vaccine, the mouse-test can be used with smaller numbers of animals in order to detect differences which, though large, are nevertheless of practical value in distinguishing between different batches of vaccine.

The eventual merit of the mouse-protection potency test is dependent upon the establishment of a close association between the results obtained in this test and those obtained in field trials. The little information so far available on this subject is that which is gradually being accumulated in the field trials that are being conducted by the MRC in Great Britain. Preliminary reports, admittedly too scanty for full interpretation, suggest that some degree of association does exist, but final evaluation of the relationship must await completion of the present studies.

The mouse-protection potency test using the intranasal instead of the intracerebral route for the challenge dose has also been used by some workers. In Australia, in particular, excellent results have been reported by this method, but there is so far a lack of confirmatory evidence. The principal advantage claimed is a steeper dosage-response curve enabling a more reliable potency estimate to be made with a given number of mice. Disadvantages include a more involved technical procedure and a lack of correlation with the results of certain field trials. It is agreed that the technique offers sufficient promise to justify further study.

The agglutination test as a measure of immune response to prophylactic immunization is considered as having some potential merit. Interpretation of the results is rendered difficult by the absence of a standard technique of performance. A reference-standard agglutinating serum would be of great value for this purpose. Accordingly, the WHO Expert Committee on Biological Standardization is asked to consider the provision of such a standard.

The conference is of the opinion that only the intracerebral mouse-protection potency test is sufficiently well established at the present time to justify its employment for the purpose of routine control. Therefore, contingent on the provision of a reference vaccine as mentioned in paragraph (3) below, the conference recommends:

1) that only those pertussis vaccines should be released for distribution which have successfully passed the mouse-protection test;

2) that the mouse-test should be performed essentially as described by the NIH 24 (see Annex 2, page 66);

3) that each batch of vaccine should be tested by direct comparison with a reference vaccine of known protective potency, as soon as one is available, and the vaccine shall be considered as having passed the test if the protection it affords is essentially equal or superior to that afforded by the reference vaccine.
The conference recommends that the WHO Expert Committee on Biological Standardization should be informed of these recommendations, and that it should be requested to consider the provision of a standard reference vaccine.

*Innocuity tests*

Both safety and toxicity tests are considered under this heading. The requirements for tests for safety and for sterility prescribed by the NIH are considered satisfactory and are recommended for general use.

The NIH regulations for a toxicity test are considered inadequate for effective control. Tests involving intravenous injection of the product into mice, tests using 3-day-old mice, and tests employing guinea-pigs, are being used in certain laboratories. However, since these techniques are in the experimental stage, it is recommended that, until a more satisfactory method is available, vaccines should be tested for toxicity by the mouse-test described in the NIH regulations (see Annex 2, page 66).

*Stability*

Available information suggests that properly prepared vaccines of high initial potency remain stable throughout extended periods of storage at refrigerator temperatures; in certain instances, no significant loss in activity has been observed during periods as long as seven to eight years. Stored at room temperature, these products have maintained a satisfactory degree of potency for as long as 21 months; and even when stored in the incubator (35°-37°C) they have retained a reasonable degree of potency for at least 6 months. Information from several sources suggests that products preserved with the usual concentrations of phenol or formalin undergo significant deterioration in potency within comparatively short periods of time. Recent observations, so far unconfirmed, indicate that a preliminary heating of vaccine either to 56°C for 30 minutes followed by the addition of 1/10,000 sodium ethylmercurithiosalicylate (merthiolate*), or to 34°C for one day after the addition of 1/5,000 merthiolate, stabilizes the product, so that the loss in activity during storage is greatly decreased.

Specific lots of pertussis vaccine killed and preserved with merthiolate have been prepared in both the liquid and the dried (lyophylized) state for stability studies in relation to reference vaccines. The dried product has been stored at room temperature and the liquid product at refrigerator temperature for a period of seven-and-a-half years. Tests for the mouse-protection potency of these preparations have been conducted at intervals

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*In certain countries the name "merthiolate" is a trademark; throughout this report, however, the term does not refer to the proprietary product.*
throughout the period of storage, and no evidence of significant decrease in protective activity of either product has been obtained. In spite of the remarkable stability of the liquid vaccine, it was considered advisable that reference vaccines should be prepared in the dried state to prevent deterioration resulting from possibly extreme environmental conditions.

The conference accepts the following requirements in regard to expiry:

(1) the date of manufacture shall be defined as the last date upon which a batch of pertussis vaccine successfully passes the intracerebral mouse-protection potency test in comparison with the reference vaccine;

(2) the permissible period of storage in the manufacturer's cold-storage room before issue for distribution without affecting the expiration date shall not exceed one year;

(3) the expiration date shall not exceed 18 months from the date of issue.

Recommendations on the production of pertussis vaccines

At present it is not known what factors are responsible for the good or poor protective activity of various vaccines. Field trials are considered to be the only means available for assessing the quality of such products. Some vaccines made by fundamentally different methods have agreed in giving good protection, whereas others made by supposedly identical methods have varied greatly in efficacy. As pointed out earlier, the intracerebral mouse-test may provide a useful means of discriminating between vaccines of satisfactory and inferior quality. The conference therefore feels that full confidence can be placed only in those products which:

(1) are produced by methods which, on the basis of adequate field trials, have been shown to be capable of yielding vaccines of good protective power, and

(2) by means of the mouse-test have been shown to possess a protective potency essentially equal or superior to a reference vaccine standardized in relation to a vaccine that has been shown to be satisfactory by adequate field trials.

The conference appreciates that only two types of vaccine have so far met these requirements. These are vaccines produced either on Bordet-Gengou solid medium and given in the fluid state, or adsorbed on to alum, or on liquid media of the casamino acid type. Our knowledge on the value of vaccines of the latter type is still scanty, but it is hoped that it will be amplified by the current British field trials.

The methods of producing and testing vaccines belonging to these two types are given in Annex 2, page 66.
Vaccine strains

The conference recommends that strains of *H. pertussis* used for the production of vaccines shall conform in general with the recognized characteristics of smooth (phase 1) strains of this species, including morphology, cultural characteristics, haemolytic activity, agglutinability with specific antisera, mouse virulence, and particularly mouse-protective activity. Inability of the strain to grow on plain nutrient agar, or on agar containing only a small proportion of blood, is considered an essential characteristic. Virulence of a vaccine strain for the mouse should be determined by direct comparison with that of a reference culture of established virulence, and the conference requests that the WHO Expert Committee on Biological Standardization shall consider the provision of such a reference challenge strain. It is recommended that strains of *H. pertussis* to be used in the production of vaccines should be maintained in the lyophilized state until activated for use, and that the culture used for the actual preparation of the vaccine should be not older than the 25th subculture from the lyophilized state. At least three or four properly characterized strains, local or otherwise, should be incorporated in each lot of vaccine.

Production medium

Media recommended for the preparation of vaccines are the Bordet-Gengou solid medium and the Cohen-Wheeler liquid medium (casamino acid type). In preparation of the Bordet-Gengou medium it is considered that the use of horse blood presents some risk of anaphylactic sensitization, and that the use of human blood incurs the possible risk of transfer of the virus of serum hepatitis. Accordingly, the use of sheep blood in a final concentration of 15%-25% by volume is recommended.

Processing details

It is recommended that the culture after inoculation should be incubated at a temperature of approximately 35°C for not more than 3 days. Cultures in liquid medium may or may not be agitated throughout the incubation period as the experience of the laboratory indicates. In cultures on solid medium the bacterial growth may be scraped off the surface and suspended in physiological saline containing the germicidal agent, or the surface growth may be washed off the medium with physiological saline and centrifuged, and resuspended in sterile saline containing the germicidal agent. Centrifugation or washing of the harvested suspension should be carried out not more than once.

The conference considers that there is some evidence that phenol or formalin may accelerate deterioration of the protective activity of the
vaccine and, accordingly, merthiolate in borate buffer is considered the chemical killing-agent of choice. It is recommended that a 1/5,000 concentration of merthiolate be employed for killing the organism and a 1/10,000 concentration for preservation. If the experience of the producing laboratory indicates that complete killing is not regularly effected within one week's storage at refrigerator temperature, the suspensions may be exposed to room temperature for one day in order to hasten the killing action.

Release of product for distribution

It is the recommendation of the conference that in no circumstances should a product be released for distribution until all tests for innocuity and protective potency have been satisfactorily completed. Since existing tests for toxicity are regarded as being inadequate, it is recommended that release of the vaccine for distribution should not be made within less than 3 months from the date of preparation.

Current research and outstanding problems

The conference agrees that problems requiring further investigation, as early and as intensively as possible, should include the following:

(1) Investigation of the nature of the immune response:
   (a) the role of agglutination and opsonocytophagic tests in assessing the efficacy of various pertussis vaccines and various immunization procedures;
   (b) the potential use of skin-test techniques for determining susceptibility to whooping cough and the adequacy of the immune response to vaccination.

(2) Methods of standardization of pertussis vaccines: particular interest is expressed in the practicability of standardizing vaccine suspensions on the basis of micro-Kjeldahl nitrogen determinations.

(3) Field trials: it is felt that there is an urgent need for adequate field trials on adsorbed vaccines, on combined vaccines (adsorbed and plain), and of new immunizing fractions (soluble vaccines).

(4) Laboratory estimation of vaccine potency:
   (a) continued research on the intracerebral mouse-protection potency test as a means of identifying superior strains of organisms for vaccine production and for the assessment of the efficacy of finished vaccines;
(b) thorough investigation and evaluation of the intranasal challenge mouse-protection potency test for the same purposes, including the rate of clearance of the organisms from the animals' lungs.

(5) Toxicity tests: thorough investigation and evaluation, in relation to possible reactions in the injected child, of certain tests proposed for determining the residual toxicity of finished vaccines including:

(a) intravenous administration to mice;
(b) use of young (3-day-old) mice;
(c) use of guinea-pigs or other test animals.

(6) Killing, preserving, and stabilizing agents: it is agreed that further information is needed on the effect on antigenicity, stability, and toxicity of various chemical and physical agents, including the possible use of new disinfectants. Continued research is encouraged, particularly in regard to:

(a) merthiolate;
(b) phenol;
(c) formalin;
(d) heating, both as a killing and as a stabilizing agent.

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Part III

COMBINED VACCINES

Laboratory trials suggest that the addition of pertussis vaccine to fluid diphtheria toxoid substantially enhances the potency of the toxoid, but that the antigenicity of the pertussis vaccine does not appear to be affected. With adsorbed diphtheria toxoid there is no evidence either in the laboratory or in the field to show that the addition of pertussis vaccine enhances its potency at all.

Preliminary observations on guinea-pigs indicate that, unless the two antigens in the mixture are correctly balanced, the one present in excess may stimulate a greater degree of immunity than the other. Much the same result has been observed when a correctly balanced mixture is given to an animal already possessing a certain degree of immunity to one of the component antigens (Barr & Llewellyn-Jones').

More information is available in both the laboratory and the field on the behaviour of diphtheria prophylactic and of pertussis vaccine when they are used separately than when they are mixed together. Until the potency of combined vaccines has been fully established by properly conducted field trials, the conference hesitates to recommend their use as freely as that of the separate vaccines.

Preparation of Combined Antigens

The conference considers that, although crude toxoid may not add noticeably to the reaction caused by a pertussis vaccine, it may cause unnecessary sensitization to foreign protein. It is therefore recommended that purified toxoids should preferably be used. In addition, it is stressed that in no circumstances should phenol be employed as a preservative for either pertussis vaccine or diphtheria toxoid, partly because of its known deleterious effect on the diphtheria-toxoid fraction, and partly because of the possibility of a similar effect on the potency of the pertussis vaccine.

The conference therefore recommends that combined vaccines should be made by one of the following methods:

(1) mixing diphtheria toxoid and pertussis vaccine, both preserved with merthiolate;

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1 Barr, M. & Llewellyn-Jones, M. (1953) Brit. J. exp. Path. 34, 12
(2) adding adsorbed diphtheria-toxoid preserved with merthiolate to pertussis vaccine, plain or adsorbed;
(3) adding purified toxoid to pertussis vaccine and adsorbing the mixture on to an alum derivative.

Pertussis organisms seem to adhere as readily to aluminium carrier particles loaded with diphtheria toxoid as to fresh alum particles. However, information is lacking as to the rate at which the two antigens are liberated from the alum when in the body. There is evidence to show that in preparations such as PTAP the union between the toxoid and aluminium phosphate becomes firmer upon standing, and that it takes up to 3 or 4 months for the bond to become complete. This raises the possibility that diphtheria toxoid may be released from its bond if another unadsorbed antigen is added in the early stages of union. It is therefore suggested that preparations of this type (PTAP) should be allowed to age before being mixed with other antigens. Further research on this subject should be undertaken.

Addition of Tetanus Toxoid

Tetanus toxoid may be added to diphtheria toxoid, or to diphtheria toxoid combined with pertussis vaccine, by the following means:

(1) mixing tetanus toxoid with diphtheria toxoid alone, or with diphtheria toxoid and pertussis vaccine;
(2) adding adsorbed tetanus-toxoid to adsorbed diphtheria-toxoid and to pertussis vaccine, plain or adsorbed;
(3) adding tetanus toxoid to diphtheria toxoid and pertussis vaccine, and adsorbing the whole on to an alum derivative.

The toxoids should preferably be of the purified type and should be preserved with merthiolate; the use of phenol should be avoided.

Observations in the Field

Reports in the literature suggest that good or better results can be obtained in immunizing against diphtheria with a mixture of fluid diphtheria-toxoid and pertussis vaccine than with fluid diphtheria-toxoid alone; the effect, however, on the development of immunity to whooping cough is far less clear.

Good results appear to be obtained with both antigens when fluid diphtheria- and tetanus-toxoids are given in combined form, but there is no evidence to show that the effect is better than when the two antigens
are given separately. Further studies are required in this respect before the effect that the antigens have on each other can be determined.

Reactions after Use of Combined Antigens

With fluid mixtures there does not appear to be an increase in either the incidence or the severity of reactions over that caused by the individual antigens given separately. This statement applies whether the prophylactics are injected subcutaneously or intramuscularly. However, with adsorbed preparations there appear to be fewer reactions after injections given by the intramuscular than by the subcutaneous route.

Under normal conditions the reactions after the injection of combined preparations, fluid or adsorbed, are not as a rule unduly severe. However, the occurrence of paralysis associated with inoculation has come to the fore within the past few years, and needs special consideration. Preliminary results in Great Britain show that, if paralytic poliomyelitis developing in the injected limb and coming on within 28 days of injection is considered alone, most cases are found to have received the combined antigen, adsorbed. Experience of post-injection paralysis varies from country to country, and has so far differed in Canada and the USA from experience in Australia and Great Britain. A special group of experts in the USA concluded that:

1. there was no definite evidence of an increase in the number of cases of poliomyelitis as a result of injection with vaccines or drugs;
2. immunizing prophylactics such as diphtheria toxoid, pertussis vaccine, and possibly tetanus toxoid may—though rarely—lead to a localization of the paralysis in the injected limb;
3. if there is any relation between inoculation and poliomyelitis, it would appear to be very slight;
4. there appears to be no reason for withholding injections in the absence of an epidemic;
5. it is advisable to continue to immunize infants under 6 months of age against pertussis, even during a poliomyelitis epidemic.

The conference feels that the effectiveness of diphtheria- and whooping-cough-immunization campaigns should be disturbed as little as possible by the fear of subsequent poliomyelitis. Immunization against diphtheria and whooping cough should normally be continued during the poliomyelitis season; but if the disease should assume serious epidemic proportions in any given area, all immunization should be temporarily suspended in that locality. If, in the opinion of the local health authority, the epidemic
is of minor severity, then immunization with diphtheria and whooping-cough vaccines may be continued, but the use of the adsorbed combined vaccine should be discouraged.

**Recommendations on the Use of Combined Vaccines**

Although, in the past, vaccination against diphtheria and pertussis has generally been carried out towards the end of the first year of life, there is at present a strong tendency in some countries to begin immunization during the early months of infancy. The advantages of starting inoculation at about the 3rd or 4th month are:

1. To protect against pertussis as early as possible. This disease has a higher case-fatality rate during the first year of life than later, approximately 70% of the deaths occurring during this period. It is hoped that by early vaccination many of these deaths may be prevented.

2. If diphtheria, and possibly tetanus, prophylactics are incorporated with the pertussis vaccine and given at the same time, the total number of injections to be given to the infant will be less than if pertussis vaccine is given separately from the other two.

3. Since poliomyelitis is uncommon during the first 6 months of life, it is maintained that vaccination starting at the 3rd month should entail practically no risk of the occurrence of this disease as a possible sequela.

On the other hand it should be remembered that the maximum incidence of both diphtheria and whooping cough occurs between one and five years of age. In some countries deaths from diphtheria during the first year of life are relatively uncommon, whereas in others they are as common as in the age-group 1-4. Although the highest case-fatality rate for pertussis occurs during the first year, it is nevertheless true to say that the majority of cases of whooping cough—possibly as many as 90%—occur after the child has reached the age of one year. Whether it is wise, therefore, in some countries to vaccinate against diphtheria and pertussis at an age when the incidence of both these diseases is low may be doubted. An immunity produced in the early months of infancy can hardly be expected to be as strong between the second and fifth years of life as an immunity resulting from vaccination at about the child's first birthday. In countries in which vaccination in early infancy is practised, a reinforcing dose of the combined vaccine is usually recommended during the second year of life in order to raise the degree of immunity sufficiently to protect the child during its period of greatest risk of infection. This practice is wholly commendable. It should, however, be pointed out that in some countries revaccination at this age presents serious practical difficulties. The children may
no longer be attending the infant-welfare centres and it is difficult to collect them for mass immunization.

There is, therefore, quite a strong body of opinion adhering to the view that immunization against diphtheria and tetanus should be delayed till towards the end of the first year of life. If vaccination against pertussis is to be practised, it may be begun in early infancy or it may be combined with diphtheria vaccination when the child is approaching the age of one year. It is argued in favour of delaying pertussis vaccination till this time that it will provide the highest degree of protection during the years of maximum exposure to risk of infection. If it is successful in this aim, the total number of children who develop whooping cough will be diminished and indirectly, therefore, the incidence of pertussis in infancy will be reduced. It is further pointed out that, although the case-fatality rate from whooping cough during infancy has in the past been fairly high, it is now less than it was. The cause of this is probably multiple, depending on better nutrition, the treatment of early cases with antibiotics, and the treatment of pulmonary complications with antibiotics and sulfonamides.

Delaying vaccination against diphtheria and pertussis till towards the end of the first year of life has the advantage, not only that protection is at its maximum during the period of greatest exposure to risk, but that the first reinforcing dose can be given when the child enters school, usually at the age of five or six years. The administrative convenience of giving the reinforcing dose at this stage, when all children are necessarily brought under medical supervision, needs no stressing.

The conference does not consider it expedient to recommend one method to the exclusion of the other. It feels rather that the choice of method must be left to individual countries. This will necessarily be influenced by the age-morbidity and -mortality figures for diphtheria and pertussis, by the current incidence of poliomyelitis, by administrative considerations, and doubtless by other factors. If vaccination during early infancy is selected, then it is strongly urged that a reinforcing dose should be given during the second year of life, followed by a further reinforcing dose when the child enters school. If, on the other hand, vaccination is practised towards the end of the first year of life, then there should be no need to give a reinforcing dose till school age is reached.

The incidence of tetanus in most temperate climates is fairly low and has little specific age distribution. Cases tend to occur at practically any time of life. Apart from tetanus neonatorum, against which vaccination of the newborn is useless, tetanus is comparatively rare during the first year of life. There is therefore no reason why antitetanus vaccination should be practised in early infancy. It should suffice to give this prophylactic agent at about the child's first birthday. After a proper course of
primary immunization, protection against tetanus is believed to last for about five years. The first reinforcing dose need not therefore be given till the child enters school. The conference stresses the importance of a true record of whether the child has been immunized against tetanus or not.

Since whooping cough diminishes greatly in incidence after the fifth year and, as a rule, becomes much less severe, there seems little point at present in revaccinating schoolchildren against this disease unless local circumstances warrant it. On the other hand, a reinforcing dose to protect against diphtheria and tetanus should preferably be given on school entry and again five years or so later. If a child who has been vaccinated or revaccinated against tetanus within the previous five years receives an injury which entails the risk of developing the disease, a single reinforcing dose of tetanus toxoid should be given as soon after the injury as possible. This will stimulate the renewed production of tetanus antitoxin and thus obviate the necessity of giving antiserum with its attendant risk of sensitization to horse serum.

It should be made clear that, if a combined vaccine containing pertussis, diphtheria, and possibly tetanus antigens is used, it should be given in three doses spaced one month apart. The reinforcing dose should consist of a single dose, equivalent as a rule to one of the individual doses used for primary immunization.

Current Research and Outstanding Problems on Combined Antigens

There is need for considerably more information on the physical state of the antigens when they are mixed together, and on their relation to the mineral carrier in adsorbed vaccines.

Further studies in both the laboratory and the field are required to determine more precisely the influence that various antigens, including tetanus, have on each other when they are combined.

Observations are especially required on the duration of immunity against each disease in infants who are immunized during the first few months of life.

Observations are likewise required on the comparative efficacy of vaccines given separately or in combination, both for primary immunization and for reinforcement of immunity.

Comparative observations are also needed on the possible effect of vaccines given separately and in combination on the predisposition to poliomyelitis, and on the incidence of paralysis in cases of poliomyelitis occurring in recently inoculated children.
Annex 1

DIPHTHERIA VACCINATION: TECHNICAL PROCEDURES

A. Essentials of the Chemistry of Alum-Precipitated Toxoid (APT)

The general method of preparing APT (Barr et al., 1 Glenny and his co-workers  2, 3) has been copied by a number of laboratories all over the world, but the products obtained have varied greatly in their antigenicity.

The expression "alum-precipitated toxoid" describes a laboratory procedure and in no way defines the nature of the material obtained either quantitatively or qualitatively.

The nature of the chemical reactions involved in alum precipitation has received attention (Glenny & Barr; 4 Linggood; 5 Holt  6).

The reactive component of alum is the aluminium sulfate moiety of the compound molecule. Crude formol-toxoid (FT), whether derived from a meat-digest medium or the relatively simple hydrolysed-casein media of Mueller & Miller, 7 is a complex mixture, that from digest media being very complex indeed. In such crude toxoids, there are a number of possibilities for chemical interaction with aluminium sulfate, the more important reactants being bicarbonate, inorganic phosphate, protein, and proteoses. The bicarbonate reacts with alum to give aluminium hydroxide, the phosphate to give aluminium phosphate, and toxoid may give aluminium toxoid (Linggood; 5 Holt 6). The proteoses present may give rise to soluble aluminium complexes, either by forming truly soluble compounds (at the pH used in the process of making APT) or by peptizing compounds otherwise insoluble. Therefore, for a given percentage of added alum, the amount of precipitate formed will depend on the quantity of peptized insoluble aluminium compounds formed. If the pH is not regulated, as by the addition of sodium hydroxide or ammonia, considerable amounts of free aluminium sulfate may be left in solution, owing to the insufficiency of reacting material.

Inorganic phosphate is always present in digest or acid hydrolysed-protein media, but usually not in a great amount. The bulk of the alum precipitate is aluminium hydroxide, together with material it has adsorbed, and organic aluminium complexes. The amount of hydroxide produced will be conditioned by the quantity of bicarbonate present in the toxoid, which may be as high as 0.3% if sodium lactate was incorporated in the original medium, and to a much less extent by the pH adopted for growing the bacillus and for the production of toxoid.

It is important to know that the natural reaction of solutions of the true alums (potassium, sodium, or ammonium alum) is about pH 2.5, and that aluminium hydroxide

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1 Barr, M., Pope, C. G., Glenny, A. T. & Linggood, F. V. (1941) Lancet, 2, 301
5 Linggood, F. V. (1939) Brit. exp. Path. 20, 502
is amphoteric with an isoelectric point of about pH 5.0 (dissociation constant about $10^{-5}$) which means that at reactions less acid than pH 5 the hydroxide acts as an acid-forming aluminate. Also, since aluminium phosphate is less soluble than the hydroxide, when an alum is added to a mixture of bicarbonate and phosphate, the phosphate will be the first to be precipitated.

A point of some importance in the preparation of APT is the choice of alum. The second sulfate, potassium, sodium, or ammonium, appears to influence the character of the precipitate produced. Potassium alum, although most commonly used, has two real disadvantages: the potassium ion modifies the adsorption characteristics of the aluminium hydroxide formed, in that nitrogenous impurities appear to be adsorbed more avidly in its presence than in its absence; and potassium ions are very irritant when inoculated, giving rise to quite severe temporary pains, followed by inflammation and oedema if much is administered. The latter fact, together with the possibility that free aluminium sulfate was present in the very early preparations of APT, probably accounts for most of the severe reactions encountered.

Concerning the possibility of the formation of aluminium protein complexes between alum and toxoid, it has been shown (Holt 8) that purified toxoid may be precipitated by very small amounts of aluminium and that the solubility of the complex is greatly influenced by the pH of the mixture. Indeed, the amount of aluminium required to precipitate the toxoid at pH 4.7 approximated very closely to the calculated theoretical amount (allowing the equivalent weight of the purified toxoid to be 1,270 g). This toxoid aluminate dissolved, on neutralizing the suspension, to give a clear solution, indicating that there was sufficient protein present to peptize the aluminium hydroxide formed.

These observations mean that it is incorrect to use the expression "alum-precipitated toxoid" when a solution of alum is added to one of purified toxoid, as either of two reactions may take place: (1) simple acid precipitation of the toxoid due to the high acidity of alum solutions, or (2) the formation of toxoid aluminate. The addition of preformed aluminium-hydroxide gel to crude FT or to a solution of purified toxoid, which is followed by adsorption of the toxoid on to the hydroxide, is the basis of the integrated diphtheria prophylactic made in Copenhagen (Schmidt & Hansen; 9 Scheibel 10).

Note: The simple addition of sterile preformed aluminium-phosphate gel to APT of small precipitate content greatly increases its antigenicity.

B. Provisions Applicable to the Reagents Used in the Schick-Test for the Diagnosis of Susceptibility to Diphtheria 11

Definitions and proper names

(1) The reagents used in the Schick-test are two, Schick toxin and Schick control. Their proper names are, respectively, "Schick-test toxoid" and "Schick-control".

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10 Scheibel, J. F. (1944) Acta path. microbiol. Scandin. 21, 150
11 Extract from United Kingdom of Great Britain and Northern Ireland (1932) Statutory rules and orders...1931 (No. 653, The therapeutic substances regulations, 1931, dated July 25, 1931, made by the Joint Committee constituted by section 4 (1) of the Therapeutic Substances Act, 1925 (15 & 16 Geo. 5. C. 60)), London, p. 1332
(2) Schick-test toxin is a sterile filtrate from a culture on nutrient broth of the specific organism of diphtheria (Corynebacterium diphtheriae). It may be issued either:

(a) undiluted, accompanied by a container in the same box or carton holding such a volume of sterile saline solution as, when mixed with the accompanying quantity of the undiluted toxin, will make a dilution of the strength proper for use in the test. The proper name of the substance in this form is "Schick-test toxin (undiluted)";

or

(b) already diluted with an appropriate saline solution to the strength proper for use in the test. The proper name of the substance in this form is "Schick-test toxin (diluted for use)".12

(3) Schick control is prepared from the same batch of Schick toxin as that with which it is used for sale, by destroying the specific toxicity. This is effected by heating the toxin in such a manner as to keep it at a temperature not lower than 70°C for a time not shorter than five minutes. Schick control is issued in a dilution not weaker than that in which the corresponding toxin is used in the test.

(4) The dilution of Schick toxin proper for the test is that in which 0.2 ml contains one test-dose.

Tests for potency

The test dose of Schick toxin for the purpose of the foregoing provision shall be measured by the following tests:

(a) by intracutaneous injection into normal guinea-pigs in mixtures with different proportions of diphtheria antitoxin. One test-dose mixed with 1,750th or more of a unit of antitoxin must cause no local reaction, but mixed with 1/1,250th or less of a unit of antitoxin must cause a definite local reaction of the type known as the "positive Schick reaction";

(b) by intracutaneous injection into normal guinea-pigs, without admixture with antitoxin. 1/50th of one test-dose must not cause, and 1/25th of one test-dose must cause, a definite local reaction of the type known as the "positive Schick reaction".

C. Technical Details for the Preparation of Diphtheria Prophylactic, Identical With, or Closely Simulating, Purified Toxoid, Aluminium-Phosphate Precipitated (PTAP)12

Part 1: Preparation of the medium, and toxin production

The medium is composed of six solutions prepared as follows:

Solution A: casein hydrolysate. To 4 litres of 8 N hydrochloric acid, analytical reagent (AR), in a 10-litre Pyrex flask add 1 kg of commercial casein. This is gently refluxed on a sand-bath for six hours. The black solution is distilled under a vacuum in lots of 1.6 litres at a time in a 5-litre round-bottomed flask. Distillation is carried out until no more acid distils off. To each lot of hot, black, viscous residue is added about 1 litre of distilled water and the residue dissolved. The distillation residues are bulked and to the acid solution, representing 1 kg of casein, is then added 10 g of pure, i.e., iron-free,

12 The stability of this preparation is under consideration by the WHO Expert Committee on Biological Standardization.

calculated oxide or hydroxide, followed by 26% sodium hydroxide (AR) until the mixture has a reaction of pH 5.0; it is then allowed to cool. To the cold, partly neutralized mixture is added good-quality decolorizing charcoal. To produce a pale-yellow filtrate we find about 300 ml of our present charcoal sufficient. By decolorizing at pH 5.0 it has been found that (a) much less charcoal is required, and (b) the iron normally present in charcoal is not dissolved by the otherwise acid mixture, and a two-stage defferruginization is not necessary. The bulk of the iron found in such mixtures originates in the charcoal used to decolorize them. The mixture is filtered through iron-free filter-paper (hydrochloric-acid-washed paper, such as Whatman no. 54, is sufficiently iron-free) into a clean bottle. The charcoal residue is emulsified twice with about 1 litre of boiling distilled water, and filtered; the filtrates are then added to the bulk. Chloroform is added as a preservative unless the next stage is carried out immediately.

To the decolorized filtrate, representing 1 kg of casein, add, previously dissolved in 300 ml of warm distilled water, 140 g of disodium hydrogen phosphate (Na₂HPO₄, 12H₂O) (AR) and 57 g of potassium dihydrogen phosphate (AR), and then 36% solution of sodium hydroxide (AR) until the mixture has a reaction of pH 7.6; the mixture is then heated to 85°C and filtered hot through iron-free filter-paper, and the precipitate is washed twice with about 500 ml of boiling distilled water; the filtrate is allowed to cool and is preserved with chloroform.

The stock hydrolysed-casein solution is analysed for nitrogen, iron, and chloride, the last-mentioned being expressed as percentage of sodium chloride. The chloride content should not greater than five times the nitrogen content. The iron content should be less than 2 µg per 10 ml; if greater than 5 µg per 10 ml, a second defferruginization must be carried out as described by Mueller.14

Solution B: mineral salt, growth-factor solution. This solution—Mueller’s solution II—is prepared exactly as described by Mueller:

- Magnesium sulfate (MgSO₄·7H₂O) .......................... 22.5 g
- β-alanine .................................................. 0.115 g
- Nicotinic acid * ........................................... 0.115 g
- Pimelic acid .............................................. 0.0073 g
- Copper sulfate (CuSO₄·5H₂O), 1% solution .............. 5.0 ml
- Zinc sulfate (ZnSO₄·7H₂O), 1% solution .................. 4.0 ml
- Manganese chloride (MnCl₂·4H₂O), 1% solution ........ 1.5 ml
- Hydrochloric acid (concentrated) ......................... 3.0 ml
- Distilled water, to make .................................. 100 ml

* Dissolve the nicotinic acid separately in about 1 ml of distilled water and a few drops of concentrated hydrochloric acid, and add to the other ingredients.

Analytical reagents must be employed, particularly in the case of the manganese chloride.

Solution C: 10% cystine solution (iron-free). 100 g of cystine are dissolved in 300 ml of warm 2.5 N hydrochloric acid (AR) and filtered into a 2.5-litre clean glass flask or bottle containing 1.5 litres of 10 N hydrochloric acid. When filtration is completed, the mixture is shaken well and left in the refrigerator overnight when the cystine hydrochloride crystallizes out. The crystals are filtered off on a sintered-glass filter and washed with about 150 ml of 10 N hydrochloric acid (AR) and sucked dry. The cystine hydrochloride is dissolved by the addition of distilled water and made up to one litre. This

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procedure gives an iron-free preparation of cystine in one stage from initially highly contaminated material. The recovery is almost quantitative.

Solution D: 15% glycine (iron-free). 500 g of synthetic glycine are dissolved in two litres of distilled water, with 10 g of calcium chloride ($\text{CaCl}_2\cdot2\text{H}_2\text{O}$) and 20 g of disodium hydrogen phosphate and the precipitate that forms is just dissolved by the addition of hydrochloric acid (AR). To the clear solution is then added 36% sodium hydroxide (AR) until a reaction of pH 7.6 is obtained. The mixture is then brought to the boil and filtered hot through iron-free filter-paper, the precipitate is washed with 500 ml of boiling distilled water, and the bulked filtrates are made up to 3.3 litres and preserved with chloroform. The solution should contain less than 2 µg of iron per 10 ml. 10 ml of this solution are used for each litre of finished medium, i.e., the latter contains 0.15% additional glycine.

Solution E: 0.01% ferrous sulfate solution. This is prepared from a stock solution of 1% ferrous sulfate in 0.1 N hydrochloric acid.

Solution F: 50% maltose solution plus 0.5% calcium chloride. It has been found that toxin production can be seriously affected unless considerable care is given to the preparation of the maltose solution. In this respect there is no doubt that prolonged heating of the maltose in alkaline solution is highly deleterious. If possible, it is preferable to use a high-grade pure maltose containing an insignificant amount of iron. When this can be done, the solution is prepared by making a 50% solution of maltose and adding to it 0.5% w/v calcium chloride ($\text{CaCl}_2\cdot2\text{H}_2\text{O}$), and is then preserved with chloroform. It is important thoroughly to saturate the solution with chloroform, otherwise moulds will grow.

If the maltose contains excessive amounts of iron, this may be removed as described by Mueller, but it is not recommended that a temperature above 50°C be used. The whole procedure should be carried out as rapidly as possible and the final iron-free maltose filtrate immediately adjusted to pH 5.0 with hydrochloric acid. Reddish solutions of maltose have invariably been found unsuitable. The calcium chloride is added after deferruginization.

An alternative method for removing iron from the maltose solution is to add to it concentrated aluminium-phosphate gel (the same gel as is used for adsorbing the purified toxoid in the final preparation of the prophylactic), to give a concentration of 2 mg of aluminium phosphate per ml. Filter through iron-free paper into acid-cleaned bottles. No adjustment of pH is required if this technique is used. The calcium chloride is added after filtration.

Culture bottles. The preparation and maintenance of the culture bottles or flasks is of first importance for the successful routine production of diphtheria toxin. Using semi-synthetic media, we have found soda-glass rectangular bottles quite satisfactory provided that they are well cared for; Thompson bottles have also been found satisfactory.

Cleaning. Bottles may be cleaned with chromic acid provided that the acid is not allowed to remain in the bottles longer than half an hour. They should then be thoroughly rinsed out with tap-water and autoclaved, filled with distilled water, or, preferably, 0.1 N hydrochloric acid (AR), for half an hour and again rinsed with distilled water. Erratic results in a batch of medium are often due to traces of chromium left in the glass after chromic-acid cleaning. Simple rinsing with water may fail to remove inhibitory amounts of the chromium. This applies, in general, to all the vessels used in the preparation of the medium. It is good laboratory practice to insist that all equipment used for the production of the medium should not be used for any other purpose.
Preparation of the medium. A 20-litre quantity of medium is prepared thus: To 10 litres of distilled water add 40 ml of solution B and solution C, followed by that amount of the stock hydrolysed-casein solution which will give 0.5% sodium chloride in the final preparation. This means that the casein nitrogen content of the medium will be approximately 0.11%. This is reinforced by the addition of 200 ml of solution D. The mixture is then made up to 20 litres with distilled water and its reaction adjusted to pH 7.6 with sodium hydroxide (AR); it is then dispensed into bottles, so that the depth of medium is 10-11 mm. The bottles are lightly plugged with cotton-wool and autoclaved for 12 minutes at a pressure of 15 pounds per square inch (about 1 kg per cm²). The amount of maltose solution to use is 2.25 ml per 100 ml of medium. The maltose solution is autoclaved separately in clean tubes, preferably with aluminium caps rather than cotton-wool plugs, and added to the medium when cold.

The amount of ferrous sulfate (solution E, diluted 1 in 100) is determined in advance, preferably using the same bottles as those employed in routine work. The results from such a trial run are plotted as a graph and, from the smooth curve drawn, the amount of iron to be added to the medium to give maximum toxin production calculated.

Since the iron content of any of the several components of the medium cannot be relied upon to remain constant from one preparation to the next, it is most essential, whenever one of the stock solutions is changed, to redetermine the optimum iron for the new group of reagents. Therefore, it is best to prepare a fair quantity of stock solutions so that it is unnecessary to make repeated optimum-iron determinations.

Strain. The strain of Corynebacterium diphtheriae found most suitable for this medium is the Toronto variant of the American PW8. This is maintained on Dorset's egg medium for intervals not exceeding three months in time. The pellicle may be used in sequence from the routine culture flasks provided that the seeding culture is not more than four days old. (Some strains of PW8 in use today have a different metabolism from that of the Toronto strain and are more suited to this medium. The present routine strain (G12/6) cannot utilize additional amino-acids of any kind, and gives the best results using 90 mg casein nitrogen per 100 ml casein hydrolysate, but requires the addition of 0.4% sodium lactate (AR, iron-free) to help regulate pH control during the growth of the organisms. 1.4% maltose in the completed medium is optimal for this strain using 90 mg % casein nitrogen. Toxin titres of 90-105 LF/ml are obtained with this strain on this medium, and a toxin purity of 1,200 LF/mg protein-nitrogen (P-N). This strain may also be preserved on Dorset's egg medium, subculturering every three months.)

Incubation. It has been found that it is best to incubate at 32°-34°C for seven days, if the medium is planted cold, or for six days if the medium has received a preliminary warming. Also, it has been found advantageous to ventilate twice daily for about 10 minutes cultures grown in box incubators.

Sterilization. The seven-day culture is filtered through tight filter-paper only; 1/10,000 ethylmercurithiosalicylate and 1/3,000 potassium iodate are added—the latter to oxidize any thiol compounds present which would inactivate the mercury preservative.

Sterility tests are carried out according to the regulations of the British Therapeutic Substances Act, 1925.

Culture notes. The toxin, after six or seven days' incubation, should be greenish-yellow in colour and have a reaction of pH 7.0-7.4. Culture filtrates more acid than pH 6.8 are indicative of excessive maltose or a deficiency of iron, and those more alkaline than pH 7.5 of a deficiency of maltose or an excess of iron.
Part 2: Conversion of toxin to toxoid

The volume of the toxin solution, which should have a titre of Lf/60-65, is measured and its amino-nitrogen content determined by the Sorensen titration technique. The result of the latter is expressed as N/10 amino-nitrogen. The toxin solution is adjusted to pH 8.4 with sodium hydroxide and 40% formaldehyde solution is added according to the following equation:

\[ \% \text{ v/v to add to the toxin} = 0.4 \times \text{amino-nitrogen value} + 0.2, \]
e.g., when the amino-nitrogen is 0.5 N/10, the amount of formalin to add is:

\[ 0.4 \times 0.5 + 0.2, \text{ which is } 0.4\% \text{ v/v.} \]

The freshly formalized toxin solution is bottled, and it is arranged that the bottles used are completely filled. These are left in a dark place at room temperature, ± 22°C, for two days, by which time the reaction has fallen to about pH 6.7. The reaction is then adjusted to pH 7.4 with sodium hydroxide, and the mixture returned to the bottles and incubated at 32°C for four weeks. By this time the formalized toxin should be completely atoxic according to the recommended innocuity tests (see page 18).

When the above technique is used, the toxoid is found to be innocuous by both innocuity tests, to have a titre of not less than 95% of the parent toxin and a Kf (using the same antitoxin and measuring conditions) of not more than three times that of the parent toxin, and should have a reaction of pH 6.6-6.8. Two notes of caution are necessary:

1. The temperature of the toxin solution should not exceed 23°C when the formalin is added;

2. When the very low nitrogen-toxins are used, the amount of formalin required is too small to act as a preservative: it is preferable to increase the amino-nitrogen of the toxin by using 5 N ammonia to adjust the toxin to pH 8.5. The Sorensen titration of this "ammoniated" toxin should be not less than 0.25 N/10. It is important to add the formalin slowly to the toxin, preferably from a slow burette when large volumes are being handled.

Part 3: Preliminary purification of FT

Batches of toxoid which have passed the innocuity tests are pooled to give a volume of 50-100 litres and the Lf, Kf, and pH noted.

To 400 ml is added 5 N ammonia to give pH 8.5, and then 8 ml of 10% magnesium sulfate (MgSO₄·7H₂O) solution. This procedure results in a greater gain in protein purity, and economizes in the use of the magnesium-hydroxide gel. To 100-ml quantities of this are then added 3, 4, 5, and 6 ml of magnesium-hydroxide gel.¹⁵

These are allowed to stand for one hour undisturbed, during which time the gel flocculates abruptly and settles as a red precipitate. The clear supernatants are decanted and neutralized with normal hydrochloric acid. The titre of each sample is then determined by the flocculation method, and that amount of magnesium-hydroxide gel is used

¹⁵ For preparation of magnesium-hydroxide gel: to a 5% solution of magnesium sulfate is added, with constant stirring, its chemical equivalent of 0.5 N sodium hydroxide; the mixture should have a reaction of pH 10. It is allowed to settle for 7-10 days and the clear supernatant withdrawn. The sedimented gel has a concentration of approximately 0.4 M. For this preparation carbon-dioxide-free water is essential.
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which, by this trial run, shows that some 10%–12% of the toxoid has been lost and all the red pigment removed.

The amount of magnesium-hydroxide gel required is usually 4% v/v. When the optimal amount of magnesium hydroxide to use has been determined, the bulk of the toxoid is placed in a large earthenware pan with a bottom outlet, into which is inserted a rubber bung, carrying two 3/8-inch (9.5-mm) tin tubes, one of which faces downwards inside the pan and the other upwards. The end of the up-pointing pipe should be about 8 cm from the bottom for a 70-litre pan. The bulk of toxoid is then adjusted to pH 8.5 with 5 N ammonia, and magnesium sulfate to give 0.2% w/v and the appropriate volume of magnesium-hydroxide gel are added; the mixture is well stirred for two minutes and left undisturbed for one hour. The clear yellow supernatant fluid is run off through the up-pointing tube and is filtered through a coarse clarifier mat (large Seitz filter). The red residue is centrifuged and the yellow supernatant added to the remainder. This is then adjusted to pH 7.5 with normal hydrochloric acid and returned to the large vessel. The titre of the filtrate should be determined at this stage, and also its protein purity if desired.

Part 4: Concentration and purification of toxoid

To the pure yellow filtrate is then added 50% cadmium chloride solution (CdCl₂·2H₂O) to give a final concentration of 0.8% w/v. The reaction of the mixture should fall to pH 6.0–6.3. This is then left for 18 hours, by which time the toxoid-protein has been precipitated. Although a further precipitation continues for as long as 48 hours afterwards, this is of no significance. The precipitated cadmium toxoid is collected by passing the whole through a Sharles centrifuge, the bowl of which is lined with cleaned x-ray film or a similar substance, such as thick cellophane. The rate at which the mixture is passed through the centrifuge should be such that a statistical millilitre remains in the bowl for a period of 10 minutes. If size No. 2A is used, the rate of flow is 35 litres per hour. The precipitated toxoid complex is then removed from the x-ray film and thoroughly emulsified in a 4% solution of K₃H₂PO₄, so that the final titre is about 10⁶. The recovery using the cadmium technique is 90%–95%.

When the cadmium toxoid is dispersed in the phosphate, its reaction is adjusted to pH 8.4 with 10% sodium hydroxide solution; this should ensure complete precipitation of all the cadmium present as the phosphate. The precipitation of the cadmium phosphate does not proceed to completion immediately, so the alkaline mixture is placed in the refrigerator for three days. By this time a clear, dark-brown supernatant on a

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15 An alternative very simple method of purifying toxoid, and one applicable to toxin produced in any medium, is as follows. Adjust the crude toxoid to pH 8.5 with dilute sodium hydroxide and then add to samples 0.0%, 0.1%, 0.25%, and 0.5% w/v sodium bicarbonate and dissolve. Then add 10% ammonia alum solution to give pH 3.8 in each case. Leave overnight, and then centrifuge. Discard the supernatants, and dissolve the precipitates in 2.5% sodium citrate, to give the original volume. Assay each sample for recovery of toxoid. From the four results obtained choose that which required the least amount of alum to give full recovery (precipitation) of the toxoid. Using these data, precipitate the batch of crude toxoid, leave overnight, aspirate clear supernatant, and centrifuge the residue. Discard the supernatant, and suspend the precipitate in 5% sodium citrate solution, so that a titre of about 10⁶ is obtained, and leave to dissolve. The reaction of the solution must be watched as it may approach pH 9.8 (namely, it may become far more alkaline than that of the citrate solution itself). The reaction of the mixture should be adjusted to between pH 8 and 8.5 as necessary, using citric acid. This sodium citrate solution of toxoid may then be fractionated with ammonium sulfate between the limits of 25% and 60% saturation.
heavy flocculent precipitate occurs. This is centrifuged, and the supernatant tested for freedom from cadmium, as indicated by the following test:

To 10 ml of the brown supernatant is added 2 ml of 25% trichloroacetic acid. After standing for five minutes, it is centrifuged and hydrogen sulfide passed through the clear supernatant, when no yellow precipitate should form. If it does, it is necessary to allow the mixture to stand a further day or two in the refrigerator. The mixture is again centrifuged and the precipitate discarded.

To the dark-brown solution of cadmium-free toxoid is added sufficient animal charcoal to remove most of the pigment and not more than 10% of the total toxoid present. This is carried out at pH 8.5. The partially decolorized solution of concentrated toxoid is then diluted to Lt 1,500, one-half of its volume of saturated ammonium sulfate solution is added, and the mixture is allowed to stand for one hour. This is then centrifuged and the precipitate discarded. The loss of toxoid in this latter precipitate should not exceed 10%. The amount of precipitated protein is very appreciable. To the clear supernatant is then added crystalline ammonium sulfate to give two-thirds saturation—250 g/litre. The mixture is filtered through paper and allowed to drain. The paper and precipitate are both placed in a cellophane dialysing bag, and dialysed against distilled water in the refrigerator for 10 days, the dialysing water being changed daily. By this time the ammonium-sulfate content in the bag should be less than 0.1% and the fluid should have a titre of Lt 3,000-6,000. This solution of concentrated purified toxoid may be freeze-dried at this stage, but if this is done the pH must first be adjusted to 7.6, otherwise the residual ammonium sulfate is likely to render the material too acid. If the concentrated toxoid solution is not freeze-dried, it is preserved by the addition of 1/1,000 merthiolate (sodium ethylmercurithiosalicylate), and sterilized by Seitz filtration.

If the very weak casein-hydrolysate medium is used (90 mg% N) the initial very high purity of toxoid present requires a slightly different treatment for complete precipitation with cadmium chloride.

The Seitz-filtered magnesium-hydroxide supernatant is adjusted to pH 8.5, and 1.6% v/v of the 50% cadmium chloride solution is added (final concentration of cadmium chloride (CdCl₂₂H₂O) 0.8%). The reaction of the mixture should be pH 7.0. Less acid mixtures result in incomplete precipitation of the toxoid.

The method of blending used in the technique for measuring the amount of toxoid present in solutions containing cadmium chloride is as follows: the usual antitoxin dilutions (1 ml or less) are placed in flocculation tubes, followed by 0.5 ml of 5% sodium citrate solution, then by 1 ml of the cadmium chloride solution, and finally by 1 ml of toxin of known titre. The citrate dissolves the cadmium phosphate that would otherwise form, owing to the phosphate in the toxin.

Part 5: Details for preparing PTAP from purified toxoid and aluminium phosphate

The purified toxoid, either in solution and preserved with merthiolate, or that supplied dry and sterile, may be used. The former is diluted to give a solution having a titre of Lt 2,400, and is sterilized by Seitz filtration. The latter is dissolved by the addition of sterile distilled water to give a solution having a titre of Lt 2,400.

Since the amount of antigen (toxoid) recommended is 15 Lt per dose, and two equal 0.5-ml doses of the completed prophylactic are advised, the titre of the finished product should be Lt 30. For human prophylaxis the optimal amount of mineral carrier (aluminium phosphate) is 5 mg, i.e., the concentration of carrier in the final prophylactic is 10 mg/ml.

It has not been found practicable to prepare a suspension of aluminium phosphate much stronger than 7 mg/ml without loss of suspension uniformity. In order, therefore,
to prepare concentrations greater than 7 mg/ml, the 7-mg/ml suspension of aluminium phosphate is allowed to stand and the amount of clear supernatant is removed to give the desired concentration. By this means it is possible, by allowing the mixture to stand for 7-10 days, to produce a preparation containing as much as 15 mg/ml.

For the preparation of PTAP having a titre of Lf 30, a mineral-carrier concentration of 10 mg of aluminium phosphate per ml, and controlled pH reaction, the following process is used. To about 50 litres of distilled water add 9.8 litres of 10% aluminium chloride (AlCl₃·6H₂O) and then slowly, with constant stirring, 15.75% sodium phosphate (Na₃PO₄·12H₂O) solution until the mixture has a reaction of pH 4.2-4.5; 9.5-9.8 litres will be required. Then add, with stirring, distilled water to give a final volume of 80 litres. This gives a suspension of 6 mg of aluminium phosphate per ml.

Cover with a dust-proof cloth, and leave to settle for seven days. Aspirate the clear supernatant to leave a residual volume of 48.25 litres. Then stir well and dispense into 4.5-litre (gallon) bottles (preferably of Pyrex glass) 3.86 litres each. This gives 12 bottles at 3.86 litres each, and one at 1.93 litres. Autoclave to sterilize for 25 minutes at a pressure of 15 pounds per square inch (about 1 kg per cm²).

It is strongly recommended that the unit volume of aluminium phosphate suspension to be autoclaved should not exceed 5 litres because, if it does, very prolonged autoclaving, which may destroy the very finely dispersed character of the gel, is necessary in order to sterilize.

The reaction of the cooled suspension is then adjusted to pH 5.2-5.5 by the aseptic addition of 5% sodium hydroxide (added with shaking); the exact amount to add is determined by titration of a one-litre sample, and is usually 20 ml for each bottle.

After adjustment of reaction, add aseptically 50 ml (per bottle of suspension) of Lf 2,400 purified toxoid, and 10 ml of 4% merthiolate—both solutions Bette-filter sterilized. A test on the centrifuged supernatant of a sample of this mixture should show complete adsorption of all the toxoid added.

The bottles of freshly prepared PTAP are then left at room temperature (temperature not exceeding 25°C) for three months. A sample adjusted to pH 6.5 and centrifuged should then show that not more than 10 Lf/ml are in solution.

The final adjustment of pH is made by adding sterile 7% sodium bicarbonate solution (sterilized by candle filtration) so that a reaction of pH 6.5 is produced; usually 0.125% sodium bicarbonate is sufficient—namely, to each bottle about 70 ml of the 7% sodium bicarbonate solution are added.

The advantages of the second adjustment of pH are twofold. The inoculation of PTAP having a reaction of pH 5.5-6 gives rise to a mild temporary stinging pain due to its acidity; this is avoided by the use of material less acid than pH 6. The thermostability of PTAP held at pH 5 is good if it is stored in the refrigerator, but very poor if it is warmed to temperatures greater than 30°C; the toxoid content gradually loses its antitoxin binding power and antigenicity. If the material is merely adjusted to pH 6.5 with sodium hydroxide, the reaction of the suspension gradually falls, particularly if it is warmed, and may return to pH 5, or become even more acid. However, by the use of sodium bicarbonate—a simple and physiological buffer—the pH is very well maintained and the prophylactic may be heated in sealed containers to 40°C for six months or more without loss of antitoxic potency, or serious destruction of the contained toxoid.

This latter advantage of the bicarbonate buffer is of no small importance in warm climates where the prophylactic may not always be stored in the refrigerator.

Note: PTAP should not be frozen as this may damage the aluminium-phosphate gel, causing it to lose much water of hydration.
D. Technical Details for the Production of a Modification of PTAP known as Phosphate Toxoid (PT) \(^7\)

**Strain**

The strain of *Corynebacterium diphtheriae* used is an American PW8 strain obtained from Dr. Levine, Massachusetts Department of Public Health, USA. It is kept in the lyophilized state. The vacuum ampoules are opened about a week before the preparation of a lot of medium and Löffler’s serum is inoculated with the strain. After two days' incubation the purity is controlled microscopically and (four to five days before the inoculation of the medium) the growth is transferred to 200-ml Erlenmeyer flasks containing about 50 ml of the fluid medium. The contents of these flasks are used as seed for toxin production.

**Medium**

75 litres of medium are prepared as follows:

- 9.5-10.5 litres of stock solution casamino acids (Difco) (the quantity is determined by the nitrogen-content; it is 10 litres if the stock solution contains 2.25% nitrogen)
- 0.7 litre of Mueller's growth-factor solution (solution B in Annex 1, C)
- 0.3 litre of 10% cystine solution (solution C in Annex 1, C)
- 3.75 litres of 50% maltose solution
- 18 ml of 50% calcium chloride (CaCl\(_2\cdot2\)H\(_2\)O) solution
- 1% solution of ferrous sulfate (FeSO\(_4\cdot7\)H\(_2\)O) may be added if necessary to give optimal iron concentration (see page 55).

The pH is adjusted to 7.2 with hydrochloric acid, and the medium is distributed into flat bottles, 300 ml per bottle, which are sterilized for 20 minutes at 120°C. They are inoculated next day.

The medium is slightly modified from that of Levine, Wyman & Edsall.\(^8\)

**Stock solutions**

Casamino acid (Difco) is dissolved in distilled water to give a concentration of 20%-22%. Usually this solution contains too much iron. It must therefore be deferrated as follows:

To 10 litres of stock solution are added 100 ml of a 50% solution of calcium chloride (CaCl\(_2\cdot2\)H\(_2\)O) and a solution of 100 g disodium hydrogen phosphate (Na\(_2\)HPO\(_4\cdot12\)H\(_2\)O). The pH is adjusted to 7.8 with concentrated sodium carbonate solution. The mixture is heated to 80°C for half an hour and left standing overnight. The calcium phosphate, which has adsorbed the iron, is then filtered off.

**Maltose\(^9\)**

A 50% solution is prepared and is deferrated in the same way except that it is heated to only 50°C instead of 80°C. The mixture is filtered immediately after heating and the pH adjusted to about 5 with hydrochloric acid.

\(^7\) Current procedure in use at the Rijks Instituut voor de Volksgezondheid, Utrecht, Holland, and developed and described by A. Tasman & J. D. van Rumsbrost


\(^9\) High-grade quality, N. V. Trifax, Bussum, Holland
Iron concentration (Tasman & van Ramshorst 39)

The optimal iron concentration is determined for each batch of stock solutions. A number of bottles containing 300 ml of medium are prepared with different iron concentrations, usually with 0.05, 1.0, 1.5, 2.0, and 2.5 ml of 0.01% FeSO₄·7H₂O solution per bottle, five or six bottles to each concentration. After five days' growth the Lf/ml and Lf/mg P-N are checked. The iron concentration chosen is that giving the highest Lf/mg P-N, though the maximal yield in terms of Lf/ml requires more iron. The purity, however, is regarded as of more importance than the quantity of the toxin.

P-N is determined in 100-ml lots of the toxin by adding 10 ml of 50% trichloroacetic acid solution, centrifuging, washing with 5% trichloroacetic acid solution, and finally determining the total nitrogen (T-N) in the precipitate by Kjeldahl analysis.

Toxin production

After inoculation the bottles are incubated for five (seldom six) days at 35°C. The toxin yield is 50-70 Lf/ml and the purity 800-1,300 Lf/mg P-N (usually about 1,000). The toxin is first freed from the gross bacterial mass by filtering through filter-paper and is then sterilized by Seitz filtration. The pH is adjusted to 7.3 with 5 N sodium hydroxide, and 0.4% formalin (40% formaldehyde) is added. After two days' storage at room temperature, the pH is adjusted to 7.1 with sodium hydroxide. After five more days' storage at room temperature the toxin is maintained at 30°C for six weeks. This results in some loss of Lf/ml, but usually the purity (Lf/mg P-N) is improved.

Innocuity test

12 guinea-pigs are injected subcutaneously with 5 ml of the crude toxoid. They are observed for eight weeks and should show no signs of diphtheria intoxication during that time.

Purification of the toxoid

The toxoid is purified by ultrafiltration followed by fractional precipitation with ammonium sulfate.

Ultrafiltration

Ultrafiltration is carried out by means of kidney-form filters covered with 8% Parlodion—a proprietary collodium—(Malinckrodt) membranes. The membranes are prepared by dipping the earthenware kidney-form filters first into water-free acetic acid, and then into an 8% solution of Parlodion in water-free acetic acid. After draining they are placed in water at 30°C for coagulation. No water must be allowed to enter the filter before complete coagulation. The filters are kept at 30°C for five hours and are then washed for two to three days in running tap-water.

The ultrafiltration is carried out by suction. A layer of toluene covers the toxoid in the ultrafiltration tanks to avoid contamination. 500-600 litres of toxoid can be ultrafiltered by means of 8-12 filters within about 14 days to a residue of only two to four litres; recovery 80%-90%.

Salting out

The residue after ultrafiltration is further purified by salting out with ammonium sulfate. The volume of the residue is made up to 10 or 15 litres with water. To about

39 Tasman, A. & Ramshorst, J. D. van (1951) Leeuwenhoek Ned. Tijdschr. 17, 153
8-10 samples of 50 ml of this solution different amounts of ammonium sulfate are added, usually 13%, 16%, 19%, 22%, and 25% at pH 7, and 22%, 25%, 28%, 31%, and 34% at pH 8. The percentages of toxoid precipitated at each concentration are calculated and the final precipitation of the bulk of the toxoid carried out as follows: first, an amount of ammonium sulfate is added (adjust to pH 7 after addition) so that about 15% of the toxoid is lost in the precipitate; then the second quantity of ammonium sulfate is added (adjust to pH 8 with ammonium hydroxide) precipitating about 70% of the toxoid and leaving about 15% in the fluid. (By increasing the losses, i.e., by choosing the limits of salting out nearer together, the purity is increased.)

The second precipitate, containing about 70% of the toxoid, is dialysed against running tap-water for five to six days in cellophane bags, the fluid inside the bags being covered with toluene.

After dialysis the toluene is filtered off using wet filter-paper, and the purified toxoid finally Seitz-filtered and merthiolate added to a concentration of 1 in 10,000. It contains 1,700-2,200Lf/mg P-N. The overall recovery of the purification process is 50%-60%.

The purified toxoid is adsorbed on aluminium phosphate as soon as possible after testing for sterility.

**Aluminium phosphate**

The aluminium-phosphate suspension is prepared by combining equivalent quantities of alum and sodium phosphate (Na₃PO₄). The precipitate is centrifuged, washed with the original volume of saline, re-centrifuged, and homogenised in saline, so that the final suspension contains 24 mg of aluminium phosphate per ml.

For the preparation of 20 litres of PT, 854 g of alum are dissolved in 6 litres of water, and 685 g of Na₃PO₄·12H₂O (or an equivalent quantity of anhydrous sodium phosphate) in 6 litres. The two solutions are poured into 21 litres of water. The volume of the final suspension is adjusted to 8.1 litres, sufficient for 20 litres of PT, containing 10 mg of aluminium phosphate per ml.

The pH of this suspension is corrected to about 6.5. The suspension is sterilised first for one hour at 100°C and immediately afterwards, after shaking, for 45 minutes at 120°C. The pH falls to about 5.8.

After cooling, 1/10,000 merthiolate is added and the flasks are kept at 37°C for three days to secure absolute sterility (the activity of the preservative is enhanced at this temperature).

**Preparation of phosphate toxoid (PT)**

The concentrated purified toxoid is mixed with the aluminium-phosphate suspension and sterile saline (containing 1/10,000 merthiolate) in such a ratio as to achieve a final product containing 30 Lf/ml and 10 mg of aluminium phosphate per ml. The mixing is carried out as far as possible under sterile conditions. 100-litre lots are mixed in a 150-litre container with an electric stirrer. The container, which is steam-heated, is first filled with 0.5% phenol and boiled for half an hour. The container is emptied and is then sterile. The saline is poured into the container, then the aluminium phosphate, and lastly the purified toxoid. These are mixed thoroughly by means of the electric stirrer. The opening in the lid is irradiated with ultraviolet light during the whole procedure of filling and emptying the container.

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31 The saline and aluminium phosphate should always be poured into the container first, as it is hot after the boiling of the phenol solution and must be cooled before the antigens are added.
The PT thus prepared is kept for three days in an incubator at 36°C to enhance adsorption and to ensure sterility.

**Routine tests**

Every batch of PT must be tested for sterility, innocuity, and potency.

The innocuity test is performed by injecting 12 guinea-pigs intraperitoneally with 5 ml of the undiluted PT. They are observed for eight weeks and should show no signs of diphtheria intoxication.

The potency is controlled by giving 12 guinea-pigs one injection of half the human dose (0.5 ml). Four weeks later the pooled sera of the animals must contain at least 2 antitoxin units per ml. Alternatively, the minimum-requirement test (see pages 17-18) may be used.

**E. Technical Details for the Preparation of Diphtheria Prophylactic, Purified Diphtheria Toxoid Adsorbed on to Aluminium Hydroxide (PTAH)**

**Strain**

The strain of *Corynebacterium diphtheriae* used is a substrain of the American PW8 strain and when grown in the medium described below produces a toxin showing a very favourable ratio of specific protein to total protein. The average ratio obtained is about 0.5, whereas other systems so far tried have given ratios of about 0.25. The term "specific protein" is based on the assumption that pure diphtheria toxin contains about 2,100 Lf/mg N.

**Medium** (based on a medium described by Pope & Linggood)

- **Pancreas extract:**
  - Ox pancreas, fresh, minced ............ 800 g
  - Tap-water .................................. 2,400 ml
  - Ethanol ................................... 1,000 ml

  Leave mixture at room temperature for three days, then add pure hydrochloric acid to a final concentration of 0.1%. The preparation may be kept for weeks in the cold.

- **Beef muscle:**
  - Beef muscle, freed of excess fat, minced .......... 8 kg
  - Tap-water ................................... 20 litres

  Heat with constant stirring to 90°C. Add 52 litres of cold tap-water, and adjust temperature to 48°-50°C and pH to about 8.0, using powdered sodium carbonate.

**Tryptic digestion**

Pancreas-extract preparation is added to media at half-hourly intervals at a rate of 600 ml for the first two additions and 300 ml subsequently; the temperature is maintained at 48°-50°C and pH at about 8.0, using 10 N sodium hydroxide, and the mixture is continuously stirred. Digestion is continued until an α-amino-acid concentration of

22 Current procedure for the preparation of PTAH in use at the Statens Seruminstitut, Copenhagen, and developed and described by I. F. Schelbel

23 Since 1950 this prophylactic has only been produced as a combined diphtheria-tetanus prophylactic

24 Pope, C. G. & Linggood, F. V. (1939) Brit. J. exp. Path. 20, 297
about 1 mg/ml is obtained. This generally occurs after five to eight additions, corresponding to a total of 2,100-3,000 ml of pancreas suspension. 1% glacial acetic acid is added, and the digest is boiled vigorously for 30 minutes, filtered through cloth bags, and stored overnight.

Next morning the filtrate is made up to a volume of 72 litres with tap-water, and heated to 30°-35°C, the pH is adjusted to 8.0 with 10 N sodium hydroxide, and 240 g of baker’s yeast emulsified in saline (0.9% sodium chloride) added. After one hour at 30°-35°C, the pH is readjusted to 8.0, the temperature brought to 80°C, 0.2% of a 50% solution of pure sodium lactate and 0.3% maltose added, and the pH again readjusted to 8.0. The broth is sterilized by filtration through a Seitz filter and distributed in suitable containers to give a layer of 0.8-1.2 cm, for example, 300-ml lots in Pliokowski bottles. Iron is added to give 0.05 mg of ferrous ions (Fe++) per litre. Bottles are steamed for 10 minutes at 100°C. The final concentration of amino-nitrogen should be 0.8-0.9 mg per ml, and T-N 2.0-3.0 mg per ml.

Inoculation and incubation

The PW8 strain is kept in lyophilized form. The contents of one ampoule are mixed in one of the medium bottles; after incubation for 24-48 hours at 32°-35°C this culture is used to inoculate the remaining bottles, approximately 1.0 ml being used for each bottle. Incubation is carried out for 7-8 days at 32°C.

Filtration

Sterile common filter-paper is used. The filtrate is shaken after the addition of toluene and stored in the coldroom. Average yield: 50-55 litres containing 50-70 Lf/ml with an average of 1,100 Lf/mg P-N.

Purification

Ultrafiltration and concentration. Large sterile Berkefeld filters or other suitable porcelain filters are coated with collodium by being gently immersed in a glass jar containing a 75%-8% solution of collodium (Parlodion) in glacial acetic acid, then placed in another sterile jar containing sterile tap-water. The water is changed after two to three hours and the filters remain in the water overnight. Next morning the water is again changed, and sterile water is sucked through the filters to remove all the acetic acid (until the pH of the filtrate is between 6 and 7).

The filters are then transferred to a suitable sterile jar provided with a mechanical stirrer. The toxin, which should be sterilized beforehand by passing it through a Seitz filter or another suitable filter, is poured into the jar. Toluene is added to ensure sterility, and the filters are connected with a vacuum pump, a container being placed between the filter and the pump to collect the filtrate. The whole procedure should take place in a cold room to prevent bacterial growth. The collodium film keeps back almost all proteins in the toxin, whereas smaller molecules pass freely. The filtrate, which thus should contain only traces of toxin, is discarded. 200-300 litres of toxin can conveniently be filtered through six to seven collodium filters of the kidney-form type in about two weeks. When all the toxin has been collected in the jar and a tenfold concentration of Lf has been obtained, the a-amino-nitrogen content is measured. If this is found to be higher

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25 Add to each 300-ml lot 0.3 ml of a solution of 0.124 g of ferrous sulfate (FeSO₄·7H₂O) and 1.2 g of sodium citrate in 500 ml of distilled water. It may be necessary to measure the iron content first before adding the Fe++, and regulate the amount to be added accordingly.
than 0.07 to 0.10 mg per 100 Lf of toxin, the filtration is continued by adding sterile saline to the concentrated toxin until the desired concentration of α-amino-nitrogen is reached.

**Detoxification.** Detoxification is carried out on the ultrafiltered, concentrated toxin. It thus becomes possible to reduce tenfold or even more the amount of formal per Lf necessary to obtain complete detoxification, since most of the amino acids that would combine with the formal in the crude toxin have been removed by the ultrafiltration. The concentrated toxin is freed from toluene by filtration through sterile common filter-paper. After determination of the T-N concentration, formal is added to give a ratio of 0.05% formal per T-N per ml of approximately 0.15 (usually, this means about 0.3% formal to a toxin of about 500-800 Lf/ml).

The pH should be about 8.5 before adding the formal, after which it should be readjusted to 7.2-7.4.

After three to four weeks' storage at 32-34°C, the toxoid is tested for toxicity by injecting 0.1 ml intradermally into rabbits or guinea-pigs. Each bottle is tested separately. If this does not give rise to any specific toxic reaction at the site of injection, the toxoid is transferred to the coldroom. Lf, Kf, and the concentrations of phosphorus and of trichloroacetic-acid-precipitable protein are assayed. Kf at this stage will usually be about 30 to 40 minutes, when the flocculation is carried out at 50°C in a dilution of about 50 Lf/ml.

**Precipitation and adsorption of non-specific proteins.** This is carried out by means of Ca++ and activated carbon: to obtain good results, which means a high degree of purity and a reasonable yield, it is essential to determine what are the optimal concentrations of such factors as are known to influence the solubility and adsorbability of proteins.

In the present system, the important factors seem to be the concentrations of (1) amino-acids, (2) calcium ions (Ca++), (3) carbon, (4) hydrogen ions (H+), (5) protein, and (6) phosphate ions (PO₄³⁻). These must be measured by preliminary tests.

(1) **Amino-acids:** A certain concentration of amino-acids in the toxoid is necessary to obtain a good degree of purification. As mentioned earlier, ultrafiltration of the toxoid is discontinued at an α-amino-nitrogen concentration of 0.07-0.1 mg per 100 Lf of toxin. This level has proved satisfactory for the purification to follow detoxification, and no further adjustment of amino-acid concentration is undertaken.

(2) **Calcium ions (Ca++):** It has been shown that minor variations in the concentration of Ca++ do not affect the process to any appreciable degree. 2% of pure calcium chloride has been found to be a suitable concentration, and may be used without preliminary test for a toxoid prepared as described.

(3) **Carbon:** Activated carbon varies widely in adsorbing capacity. It is therefore necessary to determine the optimal concentration of each new batch of carbon. Once this has been done, the same concentration may be used for most toxoids of the same type. Concentrations around 0.5% are usually satisfactory.

(4) **Hydrogen-ion concentration [pH]:** Variations in pH between 6.7 and 7.5 do not seem to influence the process to any pronounced extent, although a pH of 7.0 ± 0.2 has most often given the best results.

(5) **Protein:** The optimal protein level at which to work differs from batch to batch and should be determined by a preliminary test.

Fig. 1 shows the influence of the protein concentration on purity and yield obtained after calcium-chloride—carbon precipitation of diphtheria toxoid.

Results may vary from one preparation of toxoid to another but generally a low initial protein concentration will lead to a high degree of purity, as most of the non-specific
proteins are precipitated (or adsorbed), but at the same time the yield will be comparatively low, since more of the specific protein precipitates as well. The opposite is true for high initial protein concentrations.

(6) Phosphate ions (PO₄⁻⁻⁻): The phosphate concentration is expressed in terms of inorganic phosphorus per ml. Though the purity that can be obtained is not very much dependent on the phosphate concentration, this factor is very important for the yield. Once the protein level has been chosen it is preferable to do a preliminary test on this level with different phosphate concentrations. Fig. 2 gives an example of such a test; it shows the influence of the phosphate concentration on purity and yield obtained after calcium-chloride-carbon precipitation of diphtheria toxins on a given level of initial protein concentration.

The findings may differ from one batch to another, so that the curves are valid for one particular experiment only.

**Technique of preliminary tests.** The preliminary tests on which the results given in fig. 1 are based are described below to serve as a model for such tests.

Concentrated toxoid is diluted with saline to a series of protein concentrations. 20 ml of each concentration will be sufficient. Potassium dihydrogenphosphate is added to give a final concentration of 0.2 mg of phosphorus per ml. This is conveniently done by using a 9% solution of potassium dihydrogenphosphate (KH₂PO₄). The pH is adjusted to 7.0 with 0.1 N sodium hydroxide and 2% of calcium chloride is then added, using a 50% calcium chloride solution. After readjustment of the pH to 7.0, the activated carbon is added to a concentration of 0.5%; the pH is again adjusted to
7.0 and after 1-2 hours at room temperature each sample is filtered separately. The LF and P-N of the filtrates are determined and, on the basis of these figures, the purities and yields are calculated. The results are given in Table I; the concentrated, ultrafiltered toxoid used contained 0.77 mg P-N per ml and 1.030 Lf/ml corresponding to 1,340 Lf/mg P-N.

**Table I. Influence of protein concentration on precipitation and adsorption with calcium ions and carbon**

<table>
<thead>
<tr>
<th>mg P-N/ml</th>
<th>Lf/ml</th>
<th>Lf/mg P-N</th>
<th>mg P-N/ml</th>
<th>Lf/ml</th>
<th>Lf/mg P-N</th>
<th>Yield per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.60</td>
<td>800</td>
<td>1,340</td>
<td>0.36</td>
<td>550</td>
<td>1,520</td>
<td>72</td>
</tr>
<tr>
<td>0.46</td>
<td>610</td>
<td>1,340</td>
<td>0.21</td>
<td>380</td>
<td>1,810</td>
<td>66</td>
</tr>
<tr>
<td>0.36</td>
<td>480</td>
<td>1,340</td>
<td>0.11</td>
<td>230</td>
<td>2,000</td>
<td>50</td>
</tr>
<tr>
<td>0.28</td>
<td>370</td>
<td>1,340</td>
<td>0.07</td>
<td>160</td>
<td>2,300</td>
<td>44</td>
</tr>
</tbody>
</table>

To limit the number of preliminary tests, it is advisable to start out with at least 200-300 litres of toxoid, to ultrafilter and detoxify these in one batch, and then to carry out the preliminary investigations necessary for further purification.

**Calcium-chloride—carbon precipitation of the main bulk.** The optimal conditions found in the preliminary experiments are strictly applied when handling the main bulk. All materials and equipment should be sterilized beforehand and the whole procedure carried out under as aseptic conditions as possible to minimize the risk of contamination. The removal of the sediment may be carried out by centrifugation, but is done by us by filtration through sterile clearing sheets. After filtration a small amount of sterile saline may be passed through the clearing sheets to reduce the loss of toxoid. LF and protein content should be determined in the filtrate.

**Precipitation of the specific protein.** Under certain conditions small amounts of cadmium ions (and other divalent ions) will precipitate protein from solutions. This fact is utilized to separate the toxoid from impurities of a non-protein nature still in solution at this stage. At the same time some increase in purity can be obtained, as the solubility of proteins in cadmium solutions differs from one protein to another.

Chloride and cadmium ions must be removed before the cadmium precipitation is carried out, since the solubility of proteins in the presence of cadmium ions (Cd++) increases with increasing Cl⁻ concentration and since Ca++ would interfere with the next step, which is the elution of toxoid from the cadmium toxoid complex. Calcium chloride is most conveniently removed by ultrafiltration, as described above, except that distilled water instead of saline should be used for washing. The washing should be continued until Ca++ and Cl⁻ can no longer be demonstrated in the toxoid. At the same time the toxoid can be concentrated as will; we have found a concentration of about 1,000-2,000 Lf/ml to be most suitable. After the ultrafiltration 0.01% merthiolate may be added to the concentrated toxoid.

As a rule a final concentration of 0.007 M cadmium chloride will precipitate most of the specific protein from a toxoid solution in distilled water containing about 0.60 mg P-N/ml (this corresponds to roughly 1,000 Lf/ml, depending on the purity of the toxoid at this stage). However, we find it advantageous to determine the optimal conditions by preliminary tests. The precipitation is carried out at pH 6.0 ± 0.2. The cadmium ions are added as cadmium chloride. The precipitate is separated by centrifugation, and washed once in a cadmium chloride solution of the same molarity as was used for
precipitation. After the second centrifugation the precipitate is emulsified in a small amount of 0.15 M Na₂HPO₄. The cadmium now combines with the phosphate, whereas the toxoid goes into solution. The cadmium phosphate is then removed by centrifugation. Lf, P-N, and T-N are assayed in the supernatant. The results of a preliminary test done at a protein concentration of 0.60 mg P-N/ml with varying cadmium chloride concentrations are given in table II.

**TABLE II. EFFECT OF THE CADMIUM-ION CONCENTRATION ON PURITY AND YIELD OBTAINED AFTER CADMIUM CHLORIDE PRECIPITATION OF DIPHTHERIA TOXOID**

<table>
<thead>
<tr>
<th>Cadmium chloride (mol)</th>
<th>Before precipitation</th>
<th>After precipitation</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lf/mg T-N</td>
<td>Lf/mg P-N</td>
<td>Lf/mg T-N</td>
</tr>
<tr>
<td>0.0005</td>
<td>1,300</td>
<td>1,840</td>
<td>1,600</td>
</tr>
<tr>
<td>0.007</td>
<td>1,300</td>
<td>1,840</td>
<td>1,800</td>
</tr>
<tr>
<td>0.014</td>
<td>1,300</td>
<td>1,840</td>
<td>1,670</td>
</tr>
</tbody>
</table>

**Cadmium chloride precipitation of the main bulk.** This is carried out strictly according to the optimal conditions found by the preliminary test. All materials should be sterilized and aseptic measures followed throughout the whole procedure. By choosing an appropriate quantity of disodium hydrogen phosphate solution for the redissolution of the toxoid, a concentration of toxoid corresponding to 8,000-10,000 Lf/ml can be obtained. Thus the small amount of Cd²⁺ remaining in the toxoid solution will be of no importance whatsoever, since the toxoid is diluted about 200 times before it is used for vaccination.

Merthiolate to a concentration of 0.01% is added to the purified, concentrated toxoid. Finally the toxoid is sterilized by filtration through a Seitz filter and stored in the coldroom.

The results obtained after the different steps of one of our routine purifications are given in table III.

**TABLE III. RESULTS OBTAINED AT DIFFERENT STAGES IN THE PURIFICATION OF DIPHTHERIA TOXOID**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Lf/ml</th>
<th>Lf⁺ (min.)</th>
<th>Lf/mg T-N</th>
<th>Lf/mg P-N</th>
<th>Litres</th>
<th>Total units (millions)</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude toxin</td>
<td>60</td>
<td>12</td>
<td>33</td>
<td>900</td>
<td>535</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td>After first ultrafiltration and detoxification</td>
<td>500</td>
<td>—</td>
<td>280</td>
<td>1,100</td>
<td>54</td>
<td>27</td>
<td>85</td>
</tr>
<tr>
<td>After cadmium-ion and carbon treatment</td>
<td>225</td>
<td>—</td>
<td>—</td>
<td>1,600</td>
<td>90</td>
<td>20</td>
<td>63</td>
</tr>
<tr>
<td>After second ultrafiltration</td>
<td>1,500</td>
<td>—</td>
<td>1,125</td>
<td>1,825</td>
<td>13</td>
<td>19</td>
<td>59</td>
</tr>
<tr>
<td>After cadmium-ion precipitation and redissolution in disodium hydrogen phosphate</td>
<td>9,000</td>
<td>35</td>
<td>1,880</td>
<td>2,020</td>
<td>1.6</td>
<td>14.5</td>
<td>45</td>
</tr>
</tbody>
</table>

* Flocculation is carried out in 40 Lf/ml dilutions at 50°C.
Testing of the concentrated, purified toxoid

Sterility test: The media used for the sterility tests should contain thioglycollate to counteract the bacteriostatic effect of the merthiolate.

Inocuity tests:

(a) Dermal toxicity test. 50 Lf in 0.1 ml injected into the skin of rabbits or guinea-pigs should be tolerated without reactions.

(b) Subcutaneous toxicity test. A small sample of the main batch of concentrated, purified toxoid is made into PTAH by adsorption on to aluminium hydroxide (see below). Five times the child-dose, injected subcutaneously into five guinea-pigs, should be tolerated without reactions in any of them during an eight-week period of observation.

Antigenicity test: See the recommendations made by the diphtheria-pertussis conference, page 17.

Final preparation of PTAH

Composition of prophylactic to be used for children

- Diphtheria toxoid .................. 50 Lf/ml
- Saline ................................ q. s.
- Merthiolate .......................... 0.01%
- Aluminium hydroxide ............. 1 mg of Al per ml

Composition of prophylactic to be used for adults

- Diphtheria toxoid .................. 12.5 Lf/ml
- Saline ................................ q. s.
- Merthiolate .......................... 0.01%
- Aluminium hydroxide ............. 1 mg of Al per ml

The concentrated, purified toxoid is diluted to an Lf value about 10% higher than that required in the final vaccine. For this dilution physiological saline containing 0.01% of merthiolate is used, and the dilution must be carried out under aseptic conditions. After a sterility test, aluminium hydroxide is added as a sterile gel containing 0.8% to 1.2% of aluminium. The final concentration of aluminium in the vaccine should be 1 mg of Al per ml. (Using an aluminium-hydroxide gel containing 1% of Al, this means that 10 ml of aluminium-hydroxide gel must be added to 90 ml of the toxoid dilution.) No readjustment of the pH is necessary. When the prescriptions given are followed, the pH will be 6.8-7.0 after adding the aluminium-hydroxide gel.

The dispensing into ampoules may be done immediately after the addition of the aluminium-hydroxide gel. To ensure an even distribution of aluminium hydroxide in the toxoid during the dispensing it is necessary to stir the PTAH in the bottle from which the filling of the ampoules is done. After sealing, the ampoules are heated to 56°C for half an hour to ensure sterility. 1% of the ampoules are taken out for final sterility tests.

Dosage

The dosage recommended is two subcutaneous injections of 1 ml at an interval of four weeks and a third injection of 1 ml one year later.

A description of the preparation of the aluminium hydroxide used is given separately; see page 64.
Preparation of aluminium-hydroxide gel

The preparation of aluminium-hydroxide gel to be used for adsorption of diphtheria toxoid is not a very simple matter. A highly active and stable product can be obtained if the directions given below are carefully followed.

Freshly prepared distilled water is added to 1,917 g of ammonium alum, to a total volume of 3,750 ml. By heating the mixture the alum is dissolved. After further heating to boiling-point, the solution is very slowly cooled on a water-bath. This leads to over-saturation, but no crystallization must occur.

At the same time a solution of 550 g of ammonium sulfate in 15 litres of distilled water is prepared in a 25-litre container equipped with a slow-moving stirrer. This solution is heated over an adjustable flame to 64°C, and 2,500 ml of ammonia containing 10% NH₃ by weight are added quickly. The temperature of the mixture must be 58°C, and must be checked carefully. When the alum solution has also been cooled down to 58°C, it is quickly dumped into the ammonia—ammonium-sulfate solution. The precipitation develops heat, and after precipitation the temperature is 61°C. While the mixture is then stirred for 10 minutes, the temperature must not drop below 59°C.

During these 10 minutes a centrifuge is started and brought to full speed. The centrifuge used here (Titan, model no. 85) is of the type used for oil cleaning. Its capacity is 11.5 litres, and its heavy construction makes it well suited for our purpose. Before starting, 10 litres of distilled water, to which 86 ml of ammonia containing 10% NH₃ have been added, are filled into the centrifuge. A speed of 6,000 revolutions per minute is reached in 6 minutes, and the centrifuge is then ready to receive the contents of the container in which the precipitate has been stirred for 10 minutes. This is dumped into the centrifuge which brings down the large floccules instantaneously. The centrifuge is stopped with a special brake in less than 40 seconds. The rest of the supernatant is quickly discarded, and the sediment is transferred to a tinned-steel whipping vat, using a jet of distilled water.

The volume is brought to 35 litres by adding distilled water and 325 ml of 10% ammonia. While the suspension is being whipped rigorously for 15 minutes, the centrifuge is restarted after having been filled with water and ammonia just as before the first run. The whipped suspension is again dumped into the centrifuge, which is then quickly stopped and emptied, as before. Whipping is repeated for 15 minutes, the only difference from the first time being that now 650 ml of 10% ammonia have been added to the water. The concentration of ammonia is thus doubled and, likewise, when preparing the centrifuge for the third run, 172 ml of 10% ammonia instead of 86 ml are added to the 10 litres of distilled water.

After the third run, the sediment is whipped with 35 litres of distilled water without ammonia, and no ammonia is added to the water in the centrifuge. The washing, by whipping and centrifugation in pure distilled water, is repeated four times. At the last two centrifugations it is difficult to obtain a clear supernatant, and the suspension must therefore be filled into the centrifuge with care, in order to reduce the loss of material. The precipitate, which was white to start with, becomes yellow and wax-like during the last washings. After the last centrifugation the sediment is transferred to the whipping vat with as little water as possible, and after a homogeneous suspension has been obtained it is dumped into a stainless-steel container and autoclaved immediately at 121°C for 60 minutes. The whole procedure described is carried through in a little less than three hours.

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27 Developed and described by A. Hansen, Statens Seruminstitut, Copenhagen; available commercially
When autoclaved, the preparation consists of a homogeneous gel which contracts spontaneously, with the elimination of water. After seven days this process has come to an end, and the water is then siphoned off. The remaining gel will contain about 1.7% aluminium oxide.

As will be seen from the above description, a large amount of freshly prepared distilled water is required. It is important to remove all carbon dioxide from this water, as the presence of carbon dioxide will lower not only the yield, but also the adsorbing quality of the final gel.

A properly prepared aluminium-hydroxide gel will retain its adsorbing capacity unchanged for many years when stored in Pyrex-glass or aluminium flasks.

The adsorbing capacity of the final product may be tested by determining the fraction of diphtheria toxin which the aluminium-hydroxide gel adsorbs from a crude toxin. Such a test may be carried out as follows:

200 ml of crude diphtheria-toxin broth is adjusted to pH 6 by means of hydrochloric acid in order to avoid elution by the small amount of secondary phosphate which may be present in the crude toxin. The total volume is brought to 300 ml with distilled water, and 75 ml is filled into each of four centrifuge tubes. Four gel dilutions are made up by mixing 2.5, 5.0, 7.5, and 10 ml of the aluminium-hydroxide gel with distilled water to total volumes of 50 ml. Each of these 50-ml gel dilutions is squirted, by means of an injection syringe, into one of the centrifuge tubes. After centrifugation the fraction adsorbed can be measured, either by determining the toxicity of the supernatant in comparison with the toxicity of the crude toxin, or by eluting the toxin from the sediment, and comparing the amount of toxin thus eluted with the amount of toxin originally present, by means of flocculation. Before elution, the sediment is washed with distilled water. This is discarded by centrifugation and the sediment is mixed with 40 ml of a 2.4% sodium phosphate solution—Na₃HPO₄·12H₂O. The mixture is centrifuged and the supernatant collected, and this process is repeated with a second lot of 40 ml of sodium phosphate solution. To the pooled centrifugates distilled water is added to make the total volume 100 ml. All four adsorbates are treated in this way. By flocculation with diphtheria antitoxin we may now determine the fractions of toxin, present in the original crude toxoid, which have been recovered after adsorption and elution. From the sediment in the tube containing the largest amount of gel, at least 90% of the toxin should be recovered.
Annex 2

PERTUSSIS VACCINATION: TECHNICAL PROCEDURES

A. Mouse-Protection Test: Intracerebral Challenge

Selection and grouping of mice for test

Use white mice weighing 11-14 g, all of one sex, or with the sexes equally divided, in the several groups. Select test and control mice from the same stock to ensure comparability. For each test-antigen and for the reference antigen place in cages and label three groups of 15 mice each for immunization with three different total amounts of antigen. As control mice for each series of tests in which all the mice are to be infected at the same time, set aside four groups of 10 mice each, one group for control of the challenge dose and three groups for virulence titration of the culture.

Immunization of the test mice

The immunization schedule must be chosen to meet the particular need. In general, it should be such that the erythema dose (ED₄₀) of the reference antigen is near the middle of the immunization dosage range. The following scheme has usually been found satisfactory:

- Stock suspensions: 15,000 million per ml (15 opacity units per ml) based on photometric readings in comparison with the opacity standard
- Route: intraperitoneal
- Number of injections: one
- Total dosage, graded: 60 million, 300 million, 1,500 million respectively, for three groups of 15 mice each, for each antigen to be tested.
- Scheme for preparing antigen for injection:

<table>
<thead>
<tr>
<th>Total dose (millions)</th>
<th>Dose in 0.5-ml volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>0.5 ml of 120 million/ml</td>
</tr>
<tr>
<td>300</td>
<td>0.5 ml of 600 million/ml</td>
</tr>
<tr>
<td>1,500</td>
<td>0.5 ml of 3,000 million/ml</td>
</tr>
</tbody>
</table>

To make the suspension of 3,000 million/ml: 3.0 ml of 15,000 million/ml + 12.0 ml of saline. Make all dilutions in salt solution, 0.85%. Rest period before challenge: 14 days.

Records: prepare a chart in the record book on the day the immunization is started, to include all groups of mice, and keep a record based on daily observations beginning with the day of immunization. Summarize and estimate the 50% end-points.

Challenge of the test and control mice

(a) Challenge dose: Use a smooth culture of Haemophilus pertussis previously found in non-immunized white Swiss mice of approximately 20 g weight to have a median

1 Procedure in use at Michigan Department of Health Bureau of Laboratories, Grand Rapids, Mich., USA.
lethal dose (LD₅₀) of not more than 500-1,000 organisms when suspended in a 1% aqueous solution of casamino acids⁡ and injected intracerebrally. Culture 18,323, now in use, has an LD₅₀ of approximately 500 organisms. As one step in attempting to have uniform killing in different tests the culture is used in no less than three and no more than six subcultures from the dried state.

(b) Preparation of challenge dose:

(i) Use a culture of H. pertussis known to have an LD₅₀ of approximately 500 organisms. Using a platinum loop, harvest a 24- to 30-hour growth on Bordet-Gengou vaccine medium (which contains 1% proteose peptone) into a 1% aqueous solution of casamino acid adjusted to pH 7.0-7.2. Usually the growth from two slants 130 mm × 20 mm is harvested into 5 ml of solution, which gives ample material for standardization of the suspension and infection of 200 mice.

(ii) Screen through sterile cotton and gauze.

(iii) Check for purity and morphology by a Gram stain.

(iv) Standardize to a 5,000 million/ml suspension, based on photometer readings.

(v) Dilute the 5,000 million/ml suspension in 1% casamino acids so that 0.03 ml will contain the required dose. The following dilution table has been found helpful in making various required doses.

**SAMPLE DILUTION CHART**

*(dose in 0.03 ml)*

**Primary dilution:**

Dilute 5,000 million/ml suspension 1 : 3

Then 0.03 ml contains \( \frac{5,000,000}{100} \times \frac{1}{3} = 50,000,000 \) million

**Further dilutions:**

<table>
<thead>
<tr>
<th>Suspension used in making dilutions (organisms per 0.03 ml)</th>
<th>Dilution ratio</th>
<th>Tube no.</th>
<th>Diluted suspension (organisms per 0.03 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50,000,000</td>
<td>1 : 10</td>
<td>2</td>
<td>5,000,000</td>
</tr>
<tr>
<td>5,000,000</td>
<td>1 : 10</td>
<td>3</td>
<td>500,000</td>
</tr>
<tr>
<td>500,000</td>
<td>1 : 5</td>
<td>4</td>
<td>100,000</td>
</tr>
<tr>
<td>100,000</td>
<td>1 : 10</td>
<td>5</td>
<td>10,000</td>
</tr>
<tr>
<td>10,000</td>
<td>1 : 5</td>
<td>6</td>
<td>2,000</td>
</tr>
<tr>
<td>2,000</td>
<td>1 : 5</td>
<td>7</td>
<td>400</td>
</tr>
<tr>
<td>400</td>
<td>1 : 5</td>
<td>8</td>
<td>80</td>
</tr>
</tbody>
</table>

**Note:** As a check on the viability of the infecting dose and on the consistency of the dilutions used, colony counts are suggested. The practice at present is to plant 0.1 ml and 0.2 ml of the 80 organisms per 0.03 ml dilution on Bordet-Gengou diagnostic media.

⁡ Casamino acids (Difco technical). The salt content of this product is such that it is not necessary to add sodium chloride. If Bacto casamino acids (Difco) are used, sodium chloride must be added (4.2 g per litre to give a final chlorides test of approximately 0.6%).
medium (without peptone). This is done both before and after infection. Also as a further check on purity, 0.2 ml of the 5,000 million per ml suspension may be plated on blood-agar. The colonies are counted after incubation of the inoculated plates at 35-37°C for four days.

Because of clumping and other factors, the colony count will be considerably less than the number of organisms estimated by photometric readings. For instance, the inoculum estimated to contain 400 organisms may give a colony count of less than 200. Colony counts do not enter into the calculations but serve to give additional help in judging the validity of results.

(c) Intracerebral injection of challenge dose: No more than two-and-a-half hours should be allowed to elapse between the harvest of the challenge culture and its injection into the brain of the mouse.

Anaesthetize the mice in an atmosphere saturated with ether. The timing should be such that when one mouse is injected, another is anaesthetized. Use an 0.25-ml syringe with a 1/4-inch, 27-gauge needle. Hold the mouse's head between the thumb and forefinger, and insert the needle through the skull slightly to one side of the midline and just in front of the "hump". Only with experience will the worker learn to place the injection so that loss of animals from injury will for the most part be avoided.

(d) Course of experimental disease: Following its intracerebral injection with an infective dose of H. pertussis, the non-immunized mouse shows a series of typical symptoms. After recovery from the anaesthetic, the mouse usually appears normal for a number of days. This incubation period varies with the animal and with the dose. For example, after a dose of 100,000 organisms, it is usually four to five days before the first symptoms of illness appear; with a smaller dose the symptoms may appear later.

At first the mouse appears to be inactive, and the coat becomes ruffled. The animal is irritable and sits in a hunched position, with head lowered. After these first indications of illness, there is a progressive loss of muscular control, and avoidance of food. During the next 48 hours, the mouse becomes prostrated and shows convulsions, respiratory difficulties, and convulsions, breathing becomes laboured and death follows soon, usually after a few hours. Death seldom occurs without these typical signs of encephalitis. H. pertussis can be isolated from the brain at autopsy. Histological examination of the brain reveals brain abscess and encephalitis. There is infiltration of the choroid plexus and ependyma, with wandering cells and polymorphonuclears.

Observation period

Observe the mice daily for 14 days. Record on a protection-test chart, started on the date of infection, each death according to the day after infection of the challenge dose. Include among the deaths the mice that are paralysed on the 14th day. Deaths occurring within the first two days are not included in the calculations. Also, perform an autopsy on several mice in each series, and make a culture from the brains, to check the presence of H. pertussis.

Calculation of results

For calculation of the 50% end-point of the immunizing dose of each vaccine under test and of the killing dose of the challenge cultures, several methods are in use. The Wilson-Worcester method \(^5\) \(^4\) is suggested by the National Institutes of Health (NIH),

Tests other than mouse-protection test

(a) Tests made on bulk diluted vaccine: Sterility, mouse toxicity, and antigenicity tests are performed in accordance with NIH requirements and should be found satisfactory before the vaccine is dispensed into the final containers.

Toxicity test. At least two hours before injection, five or more mice, weighing 14-16 g each, are held in suitable cages and given adequate food and free access to water. Immediately preceding the injection they are weighed as a group and the group weight recorded. Each mouse is injected intraperitoneally with an amount of vaccine representing 7,500 million bacteria. A vaccine is considered free of toxicity if at the end of 72 hours the group weight is no less than at the time of injection, and at the end of seven days no vaccine-related deaths of mice have occurred.

(b) Test made on filled vaccine: Safety, sterility, and identity tests are performed as required by the NIH. Identity should be checked by agglutination test and Gram-stained preparation.

Safety test. A safety test shall be made on the contents of a final container selected at random from each filling of each lot or portion of a lot. The parenteral injection of the maximum volume tolerated, but not more than 0.5 ml for mice weighing approximately 20 g each and not more than 5.0 ml for guinea-pigs weighing approximately 350 g each, shall cause neither significant symptoms nor death. However, the volume given to the guinea-pig should be not less than the largest individual injection recommended for human use. At least two animals of each species are used and the observation period is not less than seven days.

B. Preparation of Pertussis Vaccine Using Potato-Infusion Blood-Agar
(Bordet-Gengou Medium)\(^{11, 15, 18, 14, 16}\)

1. Media

1.1 Bordet-Gengou diagnostic medium (BG-D),\(^{18}\) modified by Eldering & Kendrick\(^{13}\)

1.1.1 Preparation of base agar

(a) Formula:

- Peeled, sliced potatoes \( \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots 125.0 \text{ g} \)
- Glycerol, Merck's reagent \( \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots 10.0 \text{ ml} \)
- Sodium chloride (NaCl) \( \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots 5.6 \text{ g} \)
- Agar (Bacto) \( \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots 30.0 \text{ g} \)
- Distilled water \( \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots 1,000.0 \text{ ml} \)

---

\(^{1}\) United States, National Institutes of Health (1948) Minimum requirements: pertussis vaccine, Bethesda, Md.
\(^{6}\) Thompson, W. R. (1947) Bact. Rev. 11, 115
\(^{7}\) Thompson, W. R. (1950) In: Glasser, O. Medical physics, Chicago, 2, 71
\(^{10}\) Weiss, E. S. (1948) Amer. J. publ. Hlth, 38, 22
\(^{11}\) Procedure used by Michigan Department of Health Laboratories, USA

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\(^{12}\), \(^{13}\), \(^{14}\), \(^{15}\), \(^{16}\) see page 70
DIPHTHERIA AND PERTUSSIS VACCINATION

(b) Procedure (use only glass, stainless-steel, or enamelware containers):
   (i) place potatoes in gauze bag and submerge in a mixture of the glycerol and
       one-half the volume of distilled water;
   (ii) boil until the potatoes are soft—approximately 30 minutes;
   (iii) cool extract to room temperature and clarify by Sharples centrifugation
       at 35,000 revolutions per minute (r.p.m.);
   (iv) add distilled water to clarified potato extract, q.s., to original volume;
   (v) add sodium chloride and stir to dissolve;
   (vi) add agar to heated extract, stirring vigorously; heat to boiling to dissolve
       agar;
   (vii) dispense in 250-ml amounts into 16-ounce French Squares (culture bottles);
   (viii) autoclave for 25 minutes at 121°C.

(c) Checking: final pH should be 5.9-6.2.

1.1.2 Preparation of finished medium

(a) Formula:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bordet-Gengou diagnostic base</td>
<td>250.0 ml</td>
</tr>
<tr>
<td>Sterile defibrinated sheep-blood</td>
<td>50.0 ml</td>
</tr>
</tbody>
</table>

(b) Procedure:

   (i) melt base agar and cool to 50°C;
   (ii) add sheep blood aseptically (directing stream of blood against the side of
        the French Square above the agar level), and mix carefully to avoid formation
        of air bubbles;
   (iii) pour into sterile Petri plates or dispense aseptically into 6 inch × 7/8 inch
        test-tubes as required. Allow solidification to take place while tubes are in a
        slanted position.

(c) Checking:

   (i) final pH should be 7.3-7.6;
   (ii) incubate for 48 hours at 37°C to check sterility.

1.2 Bordet-Gengou vaccine medium (BG-V)12

1.2.1 Formula (for one litre):

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peeled, sliced potatoes</td>
<td>125.0 g</td>
</tr>
<tr>
<td>Glycerol, chemically pure (CP), or reagent grade</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Proteose peptone (Difco)</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sodium chloride, CP</td>
<td>5.4 g</td>
</tr>
<tr>
<td>Agar (Bacto)</td>
<td>22.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1,000.0 ml</td>
</tr>
<tr>
<td>Sterile sheep-blood</td>
<td>200.0 ml</td>
</tr>
</tbody>
</table>

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12 United States, National Institutes of Health (1948) Minimum requirements: pertussis
   vaccine, Bethesda, Md.
   P. 203
   Health Association. Diagnostic procedures and reagents, New York, p. 136
16 "D" refers to "diagnostic" medium in which there is no peptone; "V" to
   "vaccine" medium which contains peptone.
17 Pertussis vaccine medium adopted in November 1948
1.2.2 Procedure (use only glass or stainless-steel containers):

(a) Preparation of potato extract:

(i) place peeled, washed, sliced potatoes in a loose gauze bag and submerge in a mixture of glycerol and one-half the volume of distilled water;
(ii) boil gently until potatoes are soft—approximately 30 minutes;
(iii) lift out gauze bag and allow to drain, press out excess extract; force-cool extract to room temperature;
(iv) immediately clarify extract by Sharples centrifugation at about 35,000 r.p.m., using large inlet-feed nozzle;
(v) collect clarified extract and restore to original volume with distilled water.

Note: Potato extract must be used in the finished medium on the same day it is prepared. It is permissible to peel, slice, and wash the potatoes within 18 hours before processing, if they are stored dry at 0-5°C until used.

(b) Preparation of base agar:

(i) add distilled water to clarified potato extract, q.s., to original volume;
(ii) add proteose-peptone with vigorous mechanical stirring until in solution, heating towards boiling-point;
(iii) add sodium chloride with vigorous mechanical stirring until in solution;
(iv) add agar when temperature of medium has approached boiling-point (85°C). Stirring should be vigorous and care should be taken to distribute agar below the surface of the liquid. When the medium has just boiled and the agar is in solution, discontinue heating and reduce force of stirring from vigorous to gentle;
(v) adjust the pH to 7.3-7.4, using normal sodium hydroxide. Alkali should be added slowly with stirring to desired pH in such a manner that back titration is unnecessary. Since the amount of reagent required for pH adjustment will vary, apparently depending upon the age, etc., of the potatoes used, a standard amount of reagent should not be added before an initial pH reading is made.

(c) Sterilization of base medium:

(i) the hot base medium (80-95°C) is dispensed in 10-litre quantities in sterile 5-gallon (22.5-litre) Pyrex bottles: sterile dispensing assemblies are fitted to each bottle;
(ii) clamp off rubber-tubing connected to dispensing unit;
(iii) autoclave for 45 minutes at 121°C (Note: The autoclave should be pre-heated and the medium hot so that 121°C may be reached in the shortest possible time. The autoclave should be handled in such a manner that all pressure is released, and the temperature is 100°C or less, 20 minutes after sterilization is finished);
(iv) allow the medium to cool to some extent before removing from the autoclave;
(v) remove clamps attached to rubber-tubing of dispensing unit;
(vi) immediately place bottles of medium in a water-bath at 45°C. The water-bath employed should be such that a large volume of water at 45°C can be used for rapid cooling and holding of the medium at 45°C for dispensing. It has been possible to cool four to six bottles to this temperature in about one hour, by using running water at 45°C.
(d) **Preparation of finished medium:**

(i) warm sterile sheep-blood to 45°C in water-bath. This step has not been considered critical. Certainly the blood should be warm and it should not be heated above 45°C under any conditions;

(ii) add blood to medium aseptically, using positive pressure and blood-inlet siphon of medium bottle. Rotate medium bottle in water-bath continuously while blood is being added, to ensure thorough mixing.

(e) **Dispensing finished medium:**

(i) medium is dispensed by positive pressure from the medium bottle in the water-bath (Note: the air line attached to the medium bottle must pass through a tightly packed sterilizing-tube of glass wool. This container is preferably of stainless steel and about 12 inches (30 cm) long and 3 inches (7.5 cm) in diameter, tapered at each end for rubber-tubing connexions. It should be sterilized by autoclaving, together with inlet and outlet tubing, immediately before use);

(ii) dispense aseptically 90- to 100-ml quantities of medium into sterile one-litre Upjohn transfusion bottles (culture flasks). Using six to eight pounds' pressure per square inch (about 0.4-0.5 kg per cm²), 10 litres of medium can be dispensed into 100-125 bottles in 20-30 minutes;

(iii) the bottles should be placed immediately on a level surface on their sides, horizontally, for solidification. Care should be taken to keep sides and neck of bottle free from agar. Tighten Bakelite screw-caps securely.

(f) **Incubation and examination of finished medium before use:**

(i) after solidification, bottles of finished medium are incubated for 48-72 hours at 37°C;

(ii) examine each bottle macroscopically under an appropriate lamp for evidence of contamination. Discard all bottles showing even questionable evidence of contamination;

(iii) re-incubate bottles at 20°-25°C for three to four days. Examine macroscopically. Discard all contaminated bottles.

**Note:** In our experience well-trained personnel can prepare pertussis vaccine medium in quantities up to 60 litres daily with resulting contamination-rates of 5% or less for the 600-700 bottles dispensed.

1.3 Veal-Infusion agar, 2%

1.3.1 **Formula:**

Veal infusion * ........................................... 1,000.0 ml
Peptone (Bacto) ........................................... 10.0 g
Sodium chloride .......................................... 5.0 g
Agar (Bacto) .............................................. 20.0 g

* 1 pound (0.45 kg) of lean meat, ground, per 1,000 ml of distilled water

1.3.2 **Procedure:**

(a) Dissolve the ingredients in veal infusion by heating to boiling-point, stirring constantly.
(b) Adjust to pH 7.6.

(c) Boil for 10 minutes:
   (i) allow agar to stand for 1-2 minutes. If flocculation occurs, maintain at a
temperature just under the boiling-point for 5-10 minutes before filtering to
ensure complete flocculation of the precipitate;
   (ii) if flocculation has not occurred, maintain at a temperature just under the
boiling-point for 20-30 minutes. If no flocculation has occurred after standing
for 20-30 minutes, repeat from step (b).

(d) Add water to restore to original volume.

(e) Filter through cotton-gauze filter.

(f) Dispense in 300-ml amounts into 16-ounce French Squares.

(g) Autoclave for 25 minutes at 121°C. (Do not allow agar to solidify before steri-
lization.)

1.3.3 Checking:

Final pH should be 7.2-7.4.

1.4 Pittman's thiglycollate broth (for sterility tests)

1.4.1 Formula:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cystine (reagent grade)</td>
<td>0.75 g</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Dextrose (Bacto)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar (Bacto)</td>
<td>0.75 g</td>
</tr>
<tr>
<td>Yeast extract (Bacto)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Casitone</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Sodium thiglycollate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Resazurin, dissolved in water</td>
<td>0.001 g</td>
</tr>
<tr>
<td>Double-distilled water</td>
<td>1,000.0 ml</td>
</tr>
</tbody>
</table>

1.4.2 Procedure:

(a) Grind cystine in a mortar until a fine powder is obtained.
(b) Add remaining dry ingredients (except thiglycollate) and mix thoroughly.
(c) Stir in a portion of the hot water.
(d) Transfer to a steam-kettle and add remainder of water.
(e) Heat until solution is complete.
(f) Add sodium thiglycollate and stir to dissolve.
(g) Adjust to pH 8.0.
(h) Reheat but do not boil.
(i) If not clear, filter through filter-paper.
(j) Add resazurin solution and mix thoroughly.
(k) Dispense in 15-ml amounts into 6 inch × 7/8 inch tubes.
(l) Autoclave for 20 minutes at 121°C.
(m) Remove from autoclave as soon as pressure reaches zero.
1.4.3 Checking:

(a) Final pH should be 7.0-7.2.
(b) Forward six tubes to Sterility Test Department and six to Tetanus Department for checking.

Do not release lot until checks have been satisfactorily completed. Discard any lot on which checks are not satisfactory.

1.5 Physiological saline, 0.85%, unbuffered

1.5.1 Formula:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>8.3 g</td>
</tr>
<tr>
<td>Distilled water, q.s.</td>
<td>1,000.0 ml</td>
</tr>
</tbody>
</table>

1.5.2 Procedure:

(a) Dissolve sodium chloride in water.
(b) Check sodium chloride content, which should be 0.80%-0.83%, before proceeding.
(c) Filter through rayon cloth and dispense as requested.
(d) Autoclave at 121°C. The time depends on the volume in each container.

1.5.3 Checking: check for sodium chloride content, which should be 0.83%-0.88%.

1.6 Reconstituted skim-milk

1.6.1 Formula:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried skim-milk powder</td>
<td>100.0 g</td>
</tr>
<tr>
<td>Distilled water, q.s.</td>
<td>1,000.0 ml</td>
</tr>
</tbody>
</table>

1.6.2 Procedure:

(a) Stir skim milk into solution and dispense as requested (for example, in tubes of approximately 10 ml).
(b) Autoclave for 10 minutes at 115°C.

2. Strains of Haemophilus pertussis

At least four smooth, antigenically active strains of H. pertussis are used for each lot of vaccine.

2.1 Characterization of strains of H. pertussis

2.1.1 Cultural characteristics on BG-D medium

(a) Appearance of colonies—smooth, raised, glistening, pearly, almost transparent, and not over 1 mm in diameter.

(b) Zone of haemolysis—not sharply delimited but merging diffusely into the surrounding medium: usually absent when 30% or more blood is present.

2.1.2 Morphology and staining

Gram-negative coccoid bacilli occurring singly and in pairs, occasionally in short chains, practically free from pleomorphic forms.
2.1.3 Rapid agglutination-test with specific antiserum

(a) Antigen:
(i) remove growth of 36- to 72-hour culture of strain to be tested with a loop, and emulsify each loop of culture as it is added to several milliliters of saline;
(ii) screen through a thin layer of cotton;
(iii) adjust turbidity to approximately 20,000 million per ml. For standardization see discussion in section 2.8 (page 81)

(b) Antiserum:
(i) use a serum having a titre of approximately 1:5,000;
(ii) prepare a series of serum dilutions with saline, using a separate pipette for each dilution; a convenient series is as follows:

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Serum dilution required</th>
<th>Serum dilution to be used</th>
<th>Serum (ml)</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:10</td>
<td>Undiluted</td>
<td>0.3</td>
<td>2.7</td>
</tr>
<tr>
<td>2</td>
<td>1:50</td>
<td>1:10</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>1:100</td>
<td>1:50</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>1:250</td>
<td>1:100</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>1:500</td>
<td>1:100</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>1:750</td>
<td>1:100</td>
<td>0.5</td>
<td>3.25</td>
</tr>
<tr>
<td>7</td>
<td>1:1,000</td>
<td>1:100</td>
<td>0.7</td>
<td>6.3</td>
</tr>
<tr>
<td>8</td>
<td>1:1,500</td>
<td>1:1,000</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>1:2,000</td>
<td>1:1,000</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>1:2,500</td>
<td>1:1,000</td>
<td>1.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

(c) Agglutination test:
(i) into a series of agglutination tubes, pipette 0.1 ml of the serum dilutions, starting with the highest dilution; add 0.1 ml of antigen to each tube;
(ii) as control, include 0.1 ml of antigen plus 0.1 ml of saline;
(iii) shake the serum-antigen mixture by hand for 3 minutes, rocking the racks at the rate of approximately 60 back-and-forth motions per minute, and in such a way that the contents flow about three-quarters of the length up the tubes;
(iv) add 0.5 ml of saline to each tube to facilitate reading.

(d) Reading results:
(i) read immediately: tilting the tube about three inches above the concave mirror of a microscope enlarges the particles and facilitates reading;
(ii) record the titre as the highest dilution giving a 2+ reaction; this is based on the final serum dilution in the serum-antigen mixture before the addition of saline. The titre should approximate that of the serum.

2.1.4 Mouse-protection properties

Use only strains that, when killed and used as immunizing antigens, have given satisfactory potency tests.

2.1.5 Skin reactions in rabbits

(a) Prepare a suspension of 48- to 72-hour growth of H. pertussis on Bordet-Gengou medium, and standardize to 1,000 million organisms per ml; based on photometric comparisons with opacity standard.

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DIPHTHERIA AND PERTUSSIS VACCINATION

(b) Inject 0.1 ml intradermally into latero-dorsal surface of a clipped normal rabbit or guinea-pig. Several tests can be done on one rabbit, but a 5-cm space should be allowed between each test area.

c) Examine in 48-72 hours for the haemorrhagic necrosis which is characteristic of smooth strains. Occasionally a rabbit is found that does not give entirely characteristic reactions.

2.2 Maintenance of culture (maintain cultures in lyophilized form)

2.2.1 Process within three months of time of isolation or within 6 subcultures after removing from lyophile

(a) Preparation of cultures for lyophilizing:
(i) suspend 48- to 72-hour growth of H. pertussis grown on BG vaccine medium in 0.5 ml of reconstituted milk. This will make a suspension of approximately 60,000-80,000 million organisms per ml;
(ii) mix the suspension well and with a sterile capillary pipette transfer approximately 0.2-ml amounts into sterile Pyrex tubes (110 mm x 7 mm); it is important that the cotton plug fits loosely;
(iii) label each tube with H. pertussis culture number and date, and designate L1, L2, L3, etc., to indicate the number of times that the culture has been revived and redried in series;
(iv) freeze by immersing in a mixture of dry ice and alcohol. Keep in the frozen state until ready for drying.

(b) Dry the culture suspension by the lyophilize method.

(c) After the lyophilizing process, test one tube from each strain for viability.

(d) Store the tubes of dried culture at 0-5°C.

(e) Record the information of lyophile preservation on appropriate form.

2.2.2 Revive cultures from the dried state

(a) Soak tube in phenolized cotton.

(b) Holding dry tube in a piece of sterile dry gauze, scratch with sterile file. Break open tube.

(c) Resuspend, by adding sterile distilled water, saline, or broth with a pipette.

(d) Transfer to BG-D slants; if a BG-D plate is used, plant only one culture on a plate, to avoid any possibility of mixing cultures by spattering.

(e) Incubate at 35°C. Incubation period may need to be prolonged to 5-7 days for the first subculture.

2.3 Preparation of seed for vaccine production

2.3.1 Choice of cultures

(a) Use 4-6 different strains of H. pertussis which have either
(i) been isolated from whooping-cough patients within three months of the time used, or
(ii) been preserved in the lyophile state within three months of isolation (further work may show that the time of isolation is not important provided the cultures have the biological characteristics of smooth strains).
(b) Use only cultures which show all characteristics of a smooth strain (see section 2.1, page 74).

(c) Recharacterizations of the dried cultures:
(i) make complete characterization of each strain after each lyophilizing process;
(ii) make complete characterization of each strain within a year of its use for vaccine production;
(iii) test each tube recovered from lyophylite for purity and identity.

2.3.2 Handling of cultures and vaccine seed

(a) Maintenance on test-tube slants:
(i) after isolation or revival from the dried state, make one subculture of the selected culture on BG-D (BG-V may be used);
(ii) plant a large inoculum on a tube of BG-V. Repeat transfers on BG-V until growth is heavy. One or two subcultures are usually sufficient;
(iii) incubate all transfers at 35°C for from two to three days;
(iv) continue transfers on tubes of BG-V at weekly intervals to use as a source of a lower subculture when the cultures on Blakes (culture bottles) are not satisfactory for vaccine production.

(b) Maintenance on Blakes:
(i) wash growth from a tube of BG-V medium with 2-3 ml of warm sterile saline and transfer with a pipette to a one-litre Blake containing BG-V. This first growth is always scanty;
(ii) label Blake with culture and subculture number;
(iii) after incubation, wash the growth from the seed Blake with enough saline to allow about 2 ml for each Blake to be planted; seed two or three Blakes. Growth on this second Blake transfer is usually sufficiently heavy to use for seed for vaccine production. Continue the preparation of seed Blakes in this manner throughout the production season.

Note:
(1) Choose an equal number of each strain.
(2) Check each Blake microscopically and macroscopically for purity.
(3) In vaccine production, do not carry cultures beyond the 25th subculture.
(4) Discontinue production with any strain which becomes pleomorphic, or shows growth on veal-infusion agar without blood (see section 2.5.2, page 78).

2.4 Planting of cultures for vaccine production

2.4.1 Planting with sterile seed dispenser

Using a special two-way dispenser for maintaining a closed sterile system, add 50-60 ml of sterile saline to a seed culture bottle and suspend the growth by gentle rocking of the bottle. Allow the suspension to flow back into the dispenser and then dispense 1-2 ml of this suspension to each Blake to be planted.

Note: The number seeded depends on the number of Blakes available; 200 Blakes can be handled for planting or harvesting in a half-day by two people. Example: 4 cultures × 2 seed Blakes each per culture × 25 vaccine Blakes each per seed = 200 vaccine Blakes.

Use a separate seed dispenser to plant from each seed Blake.
2.4.2 Incubation

(a) Incubate Blakes in a horizontal position for 18-24 hours, then tilt Blakes so that the fluid drains to one end, and continue the incubation period for 48 hours longer in this position.

(b) The usual weekly schedule is as follows: harvest and plant on Monday; harvest on Thursday; plant on Friday. The seed for planting is prepared on Tuesday for Friday’s planting and on Friday for Monday’s planting.

2.4.3 Records

Label each vaccine Blake with a number identifying culture number, subculture number, and date planted; and record all data on inoculation of each lot.

2.5 Harvesting, purity test, centrifugation and resuspension of vaccine (operations 2.5.1-2.5.4 must be completed the same day)

2.5.1 Harvesting

(a) Inspect each Blake for purity macroscopically and discard any contaminated Blakes.

(b) Suspension of organisms on vaccine Blakes selected for harvest:

(i) by means of a sterile saline dispenser, add approximately 10 ml of sterile saline to each Blake;

(ii) suspend the growth by carefully rocking the Blakes; scraping is not recommended.

(c) Assemble a sterile harvesting unit which can be attached to a source of vacuum. A stainless-steel wire-mesh filter cylinder should be included between the suspension being harvested and the 550-ml centrifuge bottle which is used for collection of the harvest.

(d) Applying suction through a sterile pipette, remove saline suspension from each vaccine Blake. The growth from 25 Blake bottles inoculated with the same seed is pooled into the same centrifuge bottle. Sterile saline is added to bring the total volume of this pool to 500 ml. (A complete new harvest assembly is used per 25 Blake bottles to be harvested.)

(e) Filter cylinders are replaced by sterile rubber-stoppers for centrifuging.

(f) Label each centrifuge bottle with a number identifying the number of Blakes harvested, the culture number, and the subculture number.

2.5.2 Culture purity test

(a) Plant approximately 1-ml quantities of the suspension from each centrifuge bottle on each of the following:

(i) BG-V agar-slant;

(ii) yeast-infusion agar-slant;

(iii) Pittman’s thioglycollate broth. This medium serves to indicate the presence or absence of anaerobes, and observations are recorded regarding the absence of growth or gas.

(b) Incubate for 72 hours at 35°C.

(c) Inspect; if there is no contaminating organism present, the test is considered satisfactory; if contamination is present on only one medium, a retest may be performed.
2.5.3 Microscopic purity test

Examine a Gram-stained preparation made from each centrifuge bottle for both purity and smooth morphological characteristics.

2.5.4 Centrifugation

Centrifuge each bottle for three hours at 1,800-2,000 r.p.m. Using a sterile pipette, withdraw the supernatant by vacuum, discarding it into a waste flask.

2.5.5 Resuspension

(a) Resuspend the sediment in each bottle with approximately 75 ml of sterile saline containing 1/5,000 sodium ethylmercurithiosalicylate (merthiolate). Stock 1% solutions of merthiolate are made in double-distilled water stabilized by the addition of 1.4% sodium perborate; 2 ml of stock solution in 100 ml of saline give 1/5,000 merthiolate.

(b) Shake resuspended cells in a mechanical shaker for 15-20 minutes.

(c) Store in the cold until ready for further pooling.

2.5.6 Re-tests

If contamination appears on only one medium (see section 2.5.2 (page 78)) and the microscopic examination shows no contaminating organisms, suspensions may be re-tested as follows:

(a) Make a Gram-stained smear from the medium showing the contamination, and record observations.

(b) Planting re-test: Not later than one week from the time of harvest, plant 0.3-ml quantities of the resuspended material into each of three thioglycollate broth tubes. Shake thoroughly. The dilution of merthiolate in thioglycollate broth is kept above 1 in 200,000, i.e., 0.3 ml is the largest volume of a 1 in 5,000 concentration that can be added to 15 ml of broth. This 0.3 ml represents 2 ml of the uncentrifuged suspension. Immediately transfer 2-ml quantities from each thioglycollate broth into six tubes each of the three media.

(c) Incubate for 7 days at 35°C.

(d) Examine for growth. If there is no contaminating organism present, the concentrate is considered satisfactory for use in vaccine.

(e) Record results.

2.6 Pooling of centrifuged resuspended vaccine concentrates (these concentrates must be pooled within from 5 days to 4 weeks after harvest)

2.6.1 Selection of concentrates for pooling

(a) Select for pooling only those suspensions which have:

(i) satisfactory purity test (see sections 2.5.2 and 2.5.6);

(ii) no growth on veal-infusion agar without blood (see section 2.5.2);

(iii) microscopic examination showing smooth morphology (see section 2.5.3).

(b) Select concentrates to be pooled in such a manner that each pool will contain only one strain of H. pertussis.

2.6.2 Process of pooling

(a) With the apparatus and technique used for harvesting, pool the suspended cells and rinsings from centrifuge bottles into a sterile centrifuge bottle.
(b) Rinse each centrifuge bottle with 20-30 ml of sterile saline containing 1/5,000 merthiolate and add to the pool so that the volume is about 500 ml.

2.6.3 Records

(a) Label each flask with a pool number.
(b) Record details.

2.6.4 Remove samples for following tests:

(a) Density determination: agitate thoroughly and take a sample of 0.5 ml for determination of density. The purpose of this test is to obtain an estimate of the final volume of diluted vaccine. For methods see section 2.8 (page 81).

(b) Sterility tests on pools:
   (i) plant 0.3-ml samples of the pool into each of three tubes of thioglycollate broth;
   (ii) transfer immediately 2-ml quantities of this thioglycollate broth into six tubes each of:
       (1) veal-infusion agar-salt
       (2) BG-V agar-salt
       (3) thioglycollate broth;
   (iii) incubate at 35°C for 7 days;
   (iv) observe results and record; if necessary, re-tests may be performed as in section 2.5.6.

2.7 Screening (pooled vaccine is usually stored after screening until needed for dilution to final vaccine)

2.7.1 Selection of pools for screening

Choose a number of pools of the same strain which have satisfactorily passed purity test and which are equivalent to about 40 litres of finished product (usually four pools).

2.7.2 Technique of screening

(a) Have ready a sterile screening apparatus consisting of a one-gallon (4.5-litre) Pyrex bottle which is to receive the screened product, with a stopper supplied with air vent and a filter cylinder containing a stainless-steel wire screen.

(b) Operation: with a siphoning apparatus and using gravity, pass the pooled concentrate through the filter cylinder; siphon merthiolated saline into the centrifuge bottle for rinsing out the suspension, and pass the rinsings through the screening apparatus.

After all the pools and rinsings are combined, add merthiolated saline through the screening apparatus until the volume is about 3,000 ml.

2.7.3 Records

(a) Record details including: (i) screen lot-number; (ii) pools included; (iii) culture numbers included with their subculture numbers; (iv) dates of harvest; (v) date of screening; (vi) dilution factor necessary for diluting to final vaccine (this is the factor obtained after pooling); (vii) amount of merthiolate.

(b) Label the screened concentrate with a screen lot-number.
2.7.4 Remove samples from the screened lots for the following tests:

(a) Density determination, 1.0-ml sample (see section 2.8, below)

(b) Sterility test, as in section 2.5.2, page 78. (This also serves as a non-viability test of screened concentrate.)

(c) Preliminary antigenicity and toxicity tests. Screened concentrated *H. pertussis* suspensions are not used for dilution to final vaccine until they have fulfilled the requirements of these tests.

2.8 Determination of density

Standardize the density of the final product (for example to 15,000 million organisms per ml, i.e., 15 opacity units per ml) on the basis of photometric comparison with the Pyrex glass standard furnished by the NIH. (This standard was based originally on a series of counts and represented the turbidity of 10,000 million *H. pertussis* organisms per ml; it has been given the arbitrary opacity value of 10 opacity units.)

2.9 Dilution of vaccine

2.9.1 Calculation of final volume of diluted vaccine

From the density of screened concentrate, obtained at the time of pooling, calculate the total amount of diluted vaccine containing the required number of organisms, for example 15,000 million per ml, which may be prepared from the screened concentrate—i.e., if the volume of the screened concentrate is 3,000 ml and it contains 100,000 million organisms per ml, the concentrate may be diluted to 20,000 ml of vaccine containing 15,000 million organisms per ml. This would require 17,000 ml of physiological saline. In order to avoid an error in dilution, 24,000 ml of saline should be dispensed into three 3½-gallon (about 16-litre) bottles, 8,000 ml per bottle. Thus 1,500 ml of the screened concentrate can be added to each of two bottles containing 8,000 ml of saline. An additional 500 ml of saline can be added to each bottle to bring the concentration of organisms to 15,000 million per ml. The final concentration of merthiolate must be 1:10,000. The merthiolate is most conveniently added as a 1½% solution.

2.9.2 Materials

(a) 3½-gallon graduated Pyrex bottles containing about 8 litres of sterile saline. Add the calculated amount of 1½% merthiolate to each bottle before addition of cells.

(b) Several one-gallon bottles of sterile saline.

(c) Four "screens", representing four different strains of *H. pertussis*, which have satisfactorily passed tests for purity, viability, toxicity, and antigenicity. All suspensions must be shaken to a uniform consistency. This usually requires several hours' shaking on a mechanical shaker.

2.9.3 Technique of diluting

(a) Assemblage of equipment: Have ready sterile dilution apparatus. A 3½-gallon bottle containing saline to receive the diluted suspension is fitted with a stopper which contains an air bulb and a glass filter-cylinder containing a stainless-steel wire screen; a graduated dilution cylinder leads into the filter. Appropriate bulb-siphoning apparatus is needed for transferring the concentrated suspension and the saline.

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19 For instructions on testing the vaccine, see Annex 2.A, page 66.
(b) Operation: Siphon through the graduated dilution cylinder the quantity of concentrate calculated to give the required density. Siphon the calculated volume of sterile saline into the suspension and agitate the bottle thoroughly.

(c) Remove samples for:
(i) density determination: record on form provided;
(ii) antigenicity test.

Note: Time of dilution: fill into final container within three weeks of the time of diluting. Shake for two hours on day before filling and for half an hour just before filling.

2.9.3 Records

Make a record of the following details: cultures and subculture number of each (from dried state); harvest dates; date of manufacture; number of vials of each culture; concentration of preservative; volume of saline added.

Make bulk sterility test.

Note: In the above outline, it has been recognized that the apparatus for different processes must be adapted to the needs of the particular laboratory, and sketches of apparatus assemblies have been omitted. Should workers wish to have further details, it is suggested that they write to Dr. G. D. Cummings, Director, Michigan Department of Health Laboratories, Lansing 4, Michigan, USA.

C. Preparation of Pertussis Vaccine in Fluid Medium

Pertussis vaccine is prepared for prophylactic use from selected phase-I strains of known antigenic value. Cultures are grown in casein-hydrolysate medium with yeast dialysate (F29.2) (Hornibrook). The vaccine is treated with methylene blue in a final concentration of 0.01%. The finished product contains 20,000 million bacilli per ml. The immunizing activity of the vaccine is determined by protection tests in mice. Replacement of strain no. 40103 is under consideration. It is less antigenic in mice than strain no. 41405.

Strains

At present, two phase-I strains of H. pertussis are used (collection no. 40103: isolated from a cough plate taken on 14 March 1940; collection no. 41405: isolated from a cough plate taken on 5 November 1941). They were originally selected as tentative standard strains for vaccine production on the basis of their phase-I characters and antigenic activity in rabbits, and more recently have been compared with other phase-I strains in immunizing potency for mice.

These strains were both isolated at this laboratory from cases of whooping cough about two weeks after onset.

Maintenance of strains: The strains are maintained in the dry state. New dried preparations are prepared as required, usually about once in one or two years. For cultures in active use, make subcultures from dried preparations every month and maintain

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20 Current procedure in use at the Division of Laboratories and Research, New York State Department of Health and developed and described by S. M. Cohen & M. W. Wheeler


the subcultures on slants of potato-infusion rabbit-blood agar. F56B (175 mm × 22 mm tubes), with transfers at weekly intervals. Discard the weekly transfers at the end of the month, after fresh subcultures have been made from a dried preparation. To a dried culture add 0.1-0.2 ml of casein-hydrolysat medium (F29.2). Inoculate a slant and a plate of potato-infusion sheep- or rabbit-blood agar (F56A) with the culture suspension. Incubate the cultures at 34°-36°C for 48 hours or longer. When growth develops, make a slide preparation stained by Gram’s method. If there is no evidence of contamination, inoculate two or three slants of rabbit-blood agar and incubate for 48 hours. If the cultures appear pure on examination, store them in the coldroom and make fresh transfers after five days of storage.

Tests of phase-I characters: The identity and purity of the cultures are controlled by the preliminary tests made in connexion with each lot of vaccine. In addition, fresh subcultures from new lots of dried preparations are tested as follows: note the morphological and cultural properties and haemolytic activity on sheep-blood agar plates (F56A); test for agglutination with phase-I serum, virulence for mice, and capacity to induce necrotic lesions on intracutaneous injection in rabbits (see section 2.1, page 74).

Preparation of vaccine

Rooms designed for strictly aseptic technique are used for all procedures in the preparation of the vaccine. All glassware and supplies, in so far as is possible, are sterilized the day before they are needed.

A convenient schedule provides for the preparation of one lot of vaccine from 150 or more bottles of medium a week. Work with the two strains is begun during alternate weeks.

Supplies:

Media. Casein-hydrolysate medium with yeast dialysate (F29.2) dispensed in 150-ml volumes in 1-litre Pyrex Roux bottles with neck and cotton plug protected by a paper cap, and in 6-ml volumes in 175 mm × 22 mm tubes.

Salt solution. Salt solution (0.85% sodium chloride solution, F64) for diluting the vaccine is dispensed in 9-litre Pyrex bottles that contain about 400 g of glass beads.

Distilled water. Distilled water, dispensed in 1- or 2-litre amounts in 4-litre Pyrex bottles, for replacing the volume lost in the sterilization of the salt solution.

Merthiolate. A stock solution of 1% merthiolate and 1.4% sodium borate. A fresh solution is prepared each month.

Normal hydrochloric acid. For adjusting the pH of the vaccine cultures.

Bottles. Graduated 4-litre Pyrex bottles for collecting the vaccine cultures; graduated 9-litre Pyrex bottles for final pooling of the cultures of each harvest; 9-litre Pyrex bottles for diluting the vaccine.

Special outfits. Outfits for planting, harvesting, final pooling, and diluting of vaccine are used.

After use, submerge planting, harvesting, and final pooling outfits in water in covered containers for autoclaving. After sterilization, rinse the rubber-tubing in running tap-water and soak the glassware for at least half an hour in an 0.3%-0.5% hot solution of a soapless detergent. Rinse thoroughly in running water, dry in air, and reassemble. Rinse in tap-water siphons and outfits used for sterile solutions or suspensions, dry, and reassemble.

The planting outfit consists of a two-holed rubber-stopper for insertion into the vaccine-seed bottle, with an air-inlet tube and a glass-and-rubber tubing connexion to a bell-covered delivery tip.
The harvesting outfit has a two-holed rubber stopper that fits the 4-litre graduated collection bottle, with a cotton guard and glass-and-rubber tubing connexions to a glass tube tapered at the free end, which is inserted into the vaccine bottles.

The outfit for final pooling and for diluting the vaccine is one designed for a closed system of siphoning.

**Preliminary transfers—identification and purity tests:**

1st day. From a previously unopened culture of the most recent transfers of the vaccine strain, inoculate a slant of potato-infusion rabbit-blood agar, F56B (175 mm × 22 mm tube), streak a sheep-blood agar-plate (F56A), and make a slide preparation stained by Gram's method. Incubate the slant for 20-24 hours, the plate for seven days.

2nd day. Examine the slant and plate. If there is no evidence of contamination, emulsify the growth from the slant in about 1.0 ml of fluid medium (F29.2) and make a stained slide preparation. If the culture is pure and typical, inoculate two tubes of fluid medium with about 0.2 ml of culture suspension. Incubate in a slanted position for 48 hours.

4th day. Inoculate two tubes of fluid medium from each culture. Use from 0.5 to 0.75 ml of inoculum for each tube. Incubate for 48 hours in a slanted position.

6th day. Inoculate each of from 15 to 25 tubes of fluid medium, two or three more than the number of seed bottles required, with from 0.5 to 0.75 ml of culture. Incubate for 48 hours in a slanted position.

8th day. Make microscopic preparations stained by Gram's method from each tube of culture and examine. Test the agglutination reactions of the culture in one tube with phase-I serum. If the morphological and serological characters are typical, use the remaining cultures for the inoculation of fluid medium in Roux bottles to be used for seeds. Pipette the contents of one tube into each bottle. Inoculate at least one bottle more than one-tenth of the number to be planted for vaccine. Incubate the bottles, laid on their sides to give maximum surface area to volume ratio, for 72 hours.

Inoculation and incubation of vaccine cultures (11th day): Make a microscopic preparation and streak a plate from each seed bottle of culture. Discard any in which contamination is noted or suspected. Use the special planting outfit.

Connect a planting outfit to a well-shaken seed bottle, secure the stopper in the bottle with a light cord tied around the neck of the bottle, and wrap the stopper with a strip of cotton moistened with 2% cresol solution. Put a pinch-cock on the rubber-tubing that leads to the bell-covered delivery tip. Invert the seed bottle carefully so that the culture does not run into the air-inlet tube, and support the bottle on a ring stand.

Dispense the culture, in about 15-ml amounts, into 10 or 12 bottles. Mark each bottle with a number corresponding to the number of the seed culture, as well as with the strain number and date. Use a fresh planting outfit for each seed bottle. Incubate the bottles laid on their sides at 34°-36°C for 72 hours.

**Harvesting of culture (14th day)**: Examine the plates inoculated with the seed cultures and discard the bottles inoculated with any contaminated seed. Examine the remaining bottles carefully and discard those that show definite contamination. Remove samples of about 1 ml from each remaining bottle and pool the samples from bottles inoculated with the same seed. Make stained slide preparations for microscopic examination. If contamination is found or suspected in a sample pool, make stained slide preparations from each bottle in the group. Pool the cultures that appear to be pure and typical in groups of 10 or 12 to give from one-and-a-half to two litres of culture in graduated 4-litre Pyrex bottles.
Connect a harvesting outfit to a graduated 4-litre collection bottle. Protect the stopper with a cotton strip dampened in 2% cresol solution. Put a pinch-cock on the rubber-tubing. Attach the collection bottle to the vacuum line, insert the glass tube of the harvesting outfit into a vaccine culture bottle, release the pinch-cock, and draw over the culture into the collection bottle. Transfer the collecting tube to another bottle and repeat. Avoid drawing air through the outfit in withdrawing the contents of a bottle, or in transferring the collecting tube from bottle to bottle, by using the pinch-cock on the rubber-tubing.

Use a fresh harvesting outfit for each pool. From each bottle of pooled culture, with a pipette, remove a sample, streak a sheep-blood agar-plate with about 0.2 ml, and make a slide preparation. Determine the pH of each pool by means of the spot-plate method; the range may be from 7.8 to 8.4. Adjust the pH to about 7.2 with normal hydrochloric acid and shake each bottle vigorously. Record the volume of culture in each bottle and add merthiolate in a 1% solution to give a final concentration of 0.01%. Store the vaccine in the coldroom. After two days' storage, re-test each bottle of vaccine for freedom from contamination. Inoculate two tubes each containing 20 ml of sterility-test medium (F57.4) with 0.25-ml amounts of vaccine and three bottles each containing 160 ml of medium with 3- or 3.5-ml amounts and streak a sheep-blood agar-plate (F56A). Return the bottles of vaccine to the coldroom and store for at least seven days. No growth should occur in the sterility-test medium. *H. pertussis* in pure culture should develop on the plates.

**Final pooling of vaccine cultures:** From ten days to two weeks after harvesting, if the purity tests are satisfactory, pool the vaccine cultures in 9-litre graduated Pyrex bottles that contain about 400 g of glass beads. From three to five preliminary pools are collected in a 9-litre bottle. Use a closed system of siphoning and a different outfit for each final pool. For determination of the bacterial concentration, shake the bottle vigorously and thoroughly immediately before removing a sample. Transfer about 25 ml from each 9-litre bottle to a 50-ml bottle that contains glass beads. Inoculate sterility-test medium with 10 ml as before, and streak a sheep-blood agar-plate. Dilute a 25-ml sample with an equal amount of salt solution containing 0.02% merthiolate. Store the vaccine in bulk and the sample at from 4°C to 6°C. At weekly intervals, test the diluted sample for viability by inoculating a sheep-blood agar-plate (F56A) with about 0.2 ml. When the sample is non-viable, proceed with the final determination of the bacterial count and inoculate a guinea-pig with a volume of vaccine that contains about 30,000 million bacilli, as estimated by nitrogen determinations (or by turbidity standard).

**Preliminary estimation of bacterial concentration:** A preliminary estimate of the bacterial concentration of each final pool of vaccine is made by comparing the turbidity of a diluted sample with that of a glass standard that represents a vaccine containing 5,000 million bacilli per ml. Use previously selected tubes of uniform size (175 mm × 22 mm). Pipette 1 or 2 ml of the vaccine sample into a tube and add salt solution until the vaccine equals the control in density. Multiply 5,000 million by the dilution factor to obtain the estimated count.

**Final determination of bacterial concentration:** The final standardization of the vaccine is based on the bacterial Kjeldahl nitrogen content. The mean bacterial nitrogen content of 1-ml amounts of vaccine cultures containing 10,000 million bacilli has been

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28 The turbidity standard employed by the NIH corresponds to a count of approximately 10,000 million organisms per ml; it has been recommended by the WHO Expert Committee on Biological Standardization as the International Reference Preparation for Opacity (World Health Org. techn. Rep. Ser. 1953, 68).
determined for each vaccine strain. It was found to be 0.0402 mg for strain no. 40103 in ten determinations, and 0.0458 mg for strain no. 41405 in eight determinations.

**Dilution of vaccine:** Combine and dilute the final vaccine pools so that each millilitre contains 20,000 million bacilli, 10,000 million of each of the two strains. Add merthiolate to give a final concentration of 0.01%.

Up to 30 litres or more of diluted vaccine are prepared at one time. The final pools used for a diluted lot usually do not differ by more than two months in the dates of harvest. The salt solution for dilution is dispensed in 9-litre Pyrex bottles that contain about 400 g of glass beads. Determine the final volume to be contained in each bottle, usually from six to eight litres, by starting with a known volume of the final pool of one strain.

Add merthiolate to the salt solution to give a final concentration of 0.01%. The concentrated vaccines must be thoroughly shaken to ensure a uniform suspension. The glass beads in the bottles of the final pools prevent the formation of a viscous mass of sediment, but vigorous agitation of the bottles in a shaking-machine is necessary to homogenize the suspension. Use a closed system of siphoning to add the required amounts of concentrated vaccine of each strain to the salt solution.

From each bottle of a lot of diluted vaccine, take 10-ml samples for turbidity controls. In addition, remove 15- or 20-ml samples and pool them for potency and toxicity tests. Inoculate sterility-test medium and a sheep-blood agar-plate (F56A). Pipette 2 ml from each of the turbidity samples into standardized 175 mm × 22 mm tubes, dilute, and compare with a turbidity standard representing 10,000 million bacilli per ml. If there appears to be more than a 10% or 15% difference, determine the bacterial nitrogen content of the diluted vaccine and readjust if necessary. Store the vaccine and the samples in the coldroom. If *H. pertussis* develops on the plates, repeat the viability tests after a week, using the samples taken for turbidity controls. Repeat at weekly intervals until no growth occurs. When the vaccine is non-viable, proceed with the potency and toxicity tests (see Annex 2, A and page 30).

**Preparation for distribution**

Before distribution, vaccines are stored for at least four months and usually six months or more after the date of harvest. On the day before a lot of the vaccine is to be dispensed, it is thoroughly shaken in a shaking-machine for 1 1/2-1 hour to ensure a uniform suspension. It is again shaken by hand just before dispensing is begun and several times during the filling. The vaccine is dispensed in 5- and 10-ml amounts. Routine tests of sterility and harmlessness are made. A final viability test is made on a vial of the dispensed vaccine. If kept under proper conditions, it is considered satisfactory for use 18 months from the date of the last satisfactory potency test.

**Fluid medium for H. pertussis (casein-hydrolysate medium with yeast dialysate, F29.2)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casamino acids (Bacto)</td>
<td>10 g</td>
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<tr>
<td>Sodium chloride (ACS)</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate, KH₂PO₄ (ACS)</td>
<td>0.5 g</td>
</tr>
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</table>

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24 See footnotes 21 and 22, page 82.
25 With certain lots of casamino acids and for certain strains, the addition of 0.01% L-arginine and 0.005% L-histidine has been found to improve growth. Either technical or purified casamino acids may be used. Selection of a suitable lot should be made by tests for growth in small experimental batches. In addition it may be necessary to readjust the concentration of calcium chloride, iron and copper sulfates, or casamino acids.
26 A product that meets the standards of the American Chemical Society (ACS) and bears a label giving the analysis.
Magnesium chloride, MgCl₂·6H₂O (ACS)  ........ 0.4 g
Starch, soluble, powdered  ..................... 1.5 g
Calcium chloride, CaCl₂ (ACS), 1% solution  ... 1 ml
Ferrous sulfate, FeSO₄·7H₂O (ACS), 0.5% solution 2.5 ml
Copper sulfate, CuSO₄·5H₂O (ACS), 0.05% solution 1.5 ml
Cysteine hydrochloride, 1% solution  ........... 3.0 ml
Yeast dialysate  .......................... 50 ml
Distilled water, to make  ...................... 1 kg

Dissolve the casamino acids, salt, phosphate, and magnesium chloride in part of the water. Add the starch, dissolved separately by heat in part of the water, then the remaining ingredients, and make up to 1 kg. Adjust the pH to 7.2-7.3. Filter if necessary and dispense in 6-ml amounts in 175 mm × 22 mm tubes and in 150-ml amounts in 1-litre Roux bottles. Autoclave at 10 pounds per square inch (about 0.7 kg per cm²) pressure for 15 minutes.

**Yeast dialysate**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brewers' yeast (Fleischmann pure dry, type 2,019)</td>
<td>500 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>800 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2,000 ml</td>
</tr>
</tbody>
</table>

Transfer the dry yeast to a cellophane tube (500-550 mm in length, about 114 mm diameter, 0.089 mm wall-thickness). Add the 800 g of water and mix thoroughly by kneading gently. Tie each end of the tube with heavy twine, immerse it in the 2,000 ml of water in an enamelled container in such a manner that the liquid cannot spill or seep through the ends of the tube, and adjust its position to give approximate equality of liquid-level inside and outside the tube. Heat in a water-bath at 78°-80°C for seven hours. Remove the tube and transfer the dialysate, which is clear and requires no filtration, to a bottle.

**Potato-infusion blood-serum (B : with peptone) for H. pertussis (F56.A, B)**

<table>
<thead>
<tr>
<th>Component</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato infusion</td>
<td>250 g</td>
<td>250 g</td>
</tr>
<tr>
<td>Peptone (Difco proteose)</td>
<td>0 g</td>
<td>10 g</td>
</tr>
<tr>
<td>Agar (Bacto)</td>
<td>25 g</td>
<td>25 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
<td>5 g</td>
</tr>
<tr>
<td>Water</td>
<td>750 g</td>
<td>750 g</td>
</tr>
</tbody>
</table>

Sheep or rabbit blood, defibrinated, sterile ........................ 90 ml per 500 ml 90 ml per 500 ml

Dissolve the salt and agar in the water by autoclaving. Check and make up weight. Add the potato infusion (B : and peptone). Mix thoroughly, dispense as required, and autoclave for 20-30 minutes. Store. Melt the agar base as needed, cool to 45°-50°C, combine aseptically with the blood, and dispense as ordered.

**Potato infusion**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potatoes, peeled and ground</td>
<td>500 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>40 ml</td>
</tr>
<tr>
<td>Water</td>
<td>1,000 g</td>
</tr>
</tbody>
</table>
Peel the potatoes, pass through a coarse grinder, and weigh. Mix thoroughly with the water and glycerol, shake well, heat at 100°C for 60 minutes, and make up weight. Strain and squeeze gently. Allow to stand and decant clear supernatant.

*Casein-hydrolysate semi-solid agar with thioglycollate, for sterility tests (F37.4)*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar (Bacto)</td>
<td>1 g</td>
</tr>
<tr>
<td>Trypticase</td>
<td>15 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>2 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Cysteine hydrochloride</td>
<td>0.75 g</td>
</tr>
<tr>
<td>Sodium thioglycollate (Bacto)</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1,000 g</td>
</tr>
</tbody>
</table>

Resazurin, 0.1% solution * 1 ml per 1,000 g

* Freshly prepared.

Dissolve the agar in one-sixth of the water, by autoclaving. Filter through paper and place in a steam-bath. Dissolve the cysteine hydrochloride in a small amount of water, previously heated, and the remaining dry ingredients, except the thioglycollate, in another portion of the water, previously heated. Transfer to a suitable container, add the remainder of the water, and heat in a boiling-water or steam bath until the ingredients are completely dissolved. Combine with the agar, make up weight, and add the thioglycollate. Adjust to pH 7.8. The final pH after sterilization should be 7.1-7.2. Reheat on the bath for 10 minutes but do not boil. Filter through paper. Weigh the filtrate recovered and add the resazurin. Dispense as required, usually 170-ml amounts in 200-ml prescription bottles; 90-ml amounts in 100-ml French Square bottles, and 24-ml amounts in 22 mm × 175 mm tubes. Autoclave for 30 minutes. Cool promptly to 25°C in order to set the agar. Store at 20-25°C, avoiding excessive light.

Note: Under suitable conditions, the medium will usually keep for several weeks. It may be used without heating unless the upper third has become oxidized, as indicated by the pinkish colour of the resazurin at the surface. Under these circumstances it may be reheated once in a boiling-water or steam bath to drive off the adsorbed oxygen before it is used.

The sodium thioglycollate should have a purity of not less than 90% when received from the manufacturer, and each new lot should be assayed. At intervals it should be titrated for evidence of deterioration and should not be used if the percentage of \( \text{SHCH}_2\text{COONa} \) is less than 75. Store in a tightly stoppered bottle in a cool dry place protected from light. Avoid contamination with water.

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*NIH bulletin, 5 February 1946 (second revision).*