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WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Geneva. 7-13 November 1978

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WHO EXPERT COMMITTEE
ON BIOLOGICAL STANDARDIZATION

Thirtieth Report

GENERAL

The WHO Expert Committee on Biological Standardization met in Geneva from 7 to 13 November 1978. The meeting was opened on behalf of the Director-General by Dr V. Fattorusso, Director, Division of Prophylactic, Diagnostic, and Therapeutic Substances.

The Committee was informed that recent developments, at the National Institute for Biological Standards and Control, London, in the technique of weighing hygroscopic materials at very low controlled humidities, have revealed problems of unexpected magnitude in the weighing of samples of international standards.

It has been recognized for many years that the exhaustive drying applied to international standards in order to attain satisfactory stability has produced a product that may take up water rapidly from the atmosphere when the ampoule is opened, unless the material is carefully protected from ambient humidity. It has been the practice to provide, where possible, guidance on the rate of water uptake by certain international standards and reference preparations. Such guidance, however, has been based on experimental results obtained with slow weighing procedures, and new techniques have revealed that moisture uptake of 100 g/kg may occur at 50% relative humidity within two or three minutes of opening the ampoule. In the case of the proposed reference preparation of bleomycin, moisture is taken up at a rate equal to that shown by phosphorous pentoxide, at least to a content of 70 g/kg, in one minute, at 20% relative humidity. By using special equipment, which is complicated and expensive, weighings of the bleomycin preparation may be effected at relative humidities low enough to prevent undesirable errors, but such equipment is not generally available—even to many national control laboratories.

The problem may be largely avoided by distributing an international standard in freeze-dried form and assigning a defined number of international units per ampoule, thus making it unnecessary to weigh quantities of the standard preparation. The total contents of the ampoule are removed with an appropriate solvent and the final
volume is accurately adjusted. The Committee recommended that, whenever possible, future international standards and reference preparations should be prepared so as to allow the unit to be defined on the basis of the total contents of an ampoule. The Committee emphasized that, when such a procedure is used, satisfactory evidence is essential, in each case, to demonstrate that the amount of liquid filled into each ampoule does not vary by more than ±1.0% (J, page 11).

The Committee defined certain new international units on the basis of ampoule contents and recommended that the WHO International Laboratories for Biological Standards should be authorized to restate the existing definitions of international units currently expressed in weights and for which the content of international units in each ampoule is known with the necessary precision. This restatement was desirable because present weight designations may be misinterpreted to mean that a portion of material contained in the ampoule may be weighed out and represents a number of units calculable from the weight definition. This procedure is not valid and may cause large errors, since it is known that the contents of a single ampoule are not necessarily homogeneous and since the error that may occur in attempts to weigh the total contents of an ampoule by difference is likely to be significantly greater than the error (±1%) involved in distributing the liquid into the ampoules. A further advantage of the definition based only on the total contents is that particles of glass inadvertently introduced at the time of opening the ampoule will not lead to inaccuracies in use.

It is for these reasons that, for many years now, recipients of ampoules of standards that have been accurately filled have been instructed to use them on the basis of the total number or units stated to be in each ampoule. The proposed restatements of the definitions would avoid the dangers inherent in weighing, but would not alter the value of the International Unit.

In the case of a number of existing international standards and reference preparations, and future materials that cannot be freeze-dried from aqueous solution, the weight definition of the unit of activity will have to be retained. The weighing of such materials will need particular care, and the Committee recognized that the experience and equipment necessary for handling hygroscopic materials may not be available in many national control laboratories. Details of appropriate handling procedures should be made available to users.
In connexion with the Guidelines for the Preparation and Establishment of Reference Materials and Reference Reagents for Biological Substances (I, page 101) the Committee agreed that, in order to encourage the setting up of national biological reference materials, further detailed technical guidance on handling certain substances would be helpful to many laboratories, and that it would be appropriate for the WHO Secretariat to make such technical guidance widely available.

The Committee discussed the advantages of setting up international working standards (I, page 129) and agreed that the provision of working standards is advisable and especially useful in instances where the substance concerned is particularly scarce, unstable, or heterogeneous, or difficult or costly to calibrate or characterize. In the past 20 years, for example, the standardization of corticotrophin preparations had benefited considerably from the international distribution of many thousands of ampoules of a working standard of that hormone by the National Institute for Biological Standards and Control, London. At this meeting the Committee agreed that international working standards should be prepared for rabies vaccine and, if possible, for thyroid-stimulating hormone for immunoassay.

The Committee recognized that the provision of working standards on such a scale would impose a substantial financial burden on the laboratories providing them. It agreed that the principle that has been the subject of several resolutions of World Health Assemblies—that international reference materials are distributed free of charge to national authorities for the purpose of setting up national standards and to other laboratories, when national standards do not exist—should not be changed. Furthermore, the Committee agreed that the additional financial burden of storing and despatching ampoules of a working standard to all laboratories requesting such materials cannot be assumed by the International Laboratories for Biological Standards without some compensation. However, it should be clearly understood, and the recipient should be informed, that the international working standard is being provided without charge and that the recipient is being asked to reimburse only the costs involved in handling the materials. It would be left to the discretion of the individual international laboratory for biological standards to formulate the method of reimbursement.

The major activities of the Committee hitherto have been (a) the designation of international reference materials for the control of
biological substances, (b) the formulation of guidelines for the manufacture and control of biological substances administered to man, (c) the formulation of guidelines for the collection, processing, and quality control of biological reference materials, and (d) the provision of guidance on related matters. These activities have led to efficient international and national control of drugs and biological substances and have certainly made an impact on the quality of substances used for the treatment and prevention of disease. However, optimum medical treatment is based on an efficient and early clinical diagnosis as well as on the monitoring of the effect of treatment, and the results of laboratory investigations are playing an increasing role in the diagnostic process. Automation of laboratory test procedures will extend the application and importance of these functions even further, and thus the importance of the biological programme is more apparent today than it used to be. It is a vital support to medical care.

The Committee could play a more active part in this process by (a) formulating requirements for quality control of kits and reagents used in diagnostic procedures, (b) giving guidance on quality control and assessment of performance in health laboratories, and (c) drawing up guidelines for the preparation of reference materials (suitable for the routine measurement of components of body fluids) calibrated against international standards appropriate for the implementation of such quality control of diagnostic assays and tests.

The assurance of acceptable quality, batch by batch, at both the national and international levels is certainly having an immense impact on the improvement of medical care.

The Committee was informed that the use of binding assay systems is developing rapidly and in many instances tending to replace the use of bioassays. Indeed a number of international standards and reference preparations for immunoassays have already been established. The Committee agreed that it may be useful to study selected bioassay standards to determine their suitability for use as standards for immunoassay. It recommended, furthermore, that, in collaborative studies for the establishment of standards for bioassay, binding assays should be included where possible to check the suitability of the materials to serve also as standards for immunoassays.

With regard to the revised requirements for diphtheria, pertussis, and tetanus (DPT) vaccines, the Committee was informed of the need for immunization schedules using only two doses of vaccines, rather
than three, for the protection of children against infectious diseases, particularly in the developing world. This is most important for countries in which it is almost impossible to reach the children more than twice a year. However, it is also important, before adopting such schedules, to carry out studies to demonstrate their efficacy.

Almost all the data available throughout the world on the efficacy of vaccines refer to the use of three doses of DPT and poliomyelitis (oral). Furthermore, most of these data emanate from studies in children in developed countries.

Studies concerning two-dose schedules, under way in the developing countries, are giving encouraging results, but special vaccines containing higher concentrations of antigens and adjuvants than those in general use are being used. The data from such studies, therefore, must be interpreted with caution and do not apply to the vaccines referred to in the Requirements for Biological Substances. The Committee recommended that health authorities in the developing countries should evaluate data on vaccine efficacy from their own communities before adopting two-dose schedules using vaccines of the potency specified in the WHO Requirements (see Annex 5).

SUBSTANCES

ANTIBIOTICS

1. Candidin

The Committee noted the report (2) of the collaborative assay of the proposed international reference preparation of candidin and noted that, in accordance with the authorization in its twenty-ninth report (1, page 11), the National Institute for Biological Standards and Control, London, has established the preparation as the International Reference Preparation of Candidin and, on the basis of the results and with the agreement of the participants, has defined the International Unit of Candidin as the activity contained in 0.000476 mg of the International Reference Preparation of Candidin (2).

The Committee noted also that the hygroscopic nature of the materials made weighing of the samples difficult (2).
2. Erythromycin

The Committee noted the report (3) of the collaborative assay referred to in its twenty-ninth report (I, page 10). It noted also that, in accordance with the authorization in that report, the National Institute for Biological Standards and Control, London, on the basis of the results of the collaborative assay, has established the Second International Standard for Erythromycin and, with the agreement of the participants, has defined the International Unit for Erythromycin as the activity contained in 0.001087 mg of the Second International Standard for Erythromycin (3).

3. Streptomycin

The Committee was informed that the results from all the participants in the collaborative assay, referred to in its twenty-ninth report (I, page 10), of the proposed third international standard for streptomycin have been received.

It was informed also that, in accordance with the authorization in its twenty-ninth report (I, page 10), the National Institute for Biological Standards and Control, London, will establish the preparation as the third international standard for streptomycin and, on the basis of the results and with the agreement of the participants, will define the international unit.

4. Amikacin

The Committee was informed that, in accordance with the request made in its twenty-ninth report (I, page 12), the National Institute for Biological Standards and Control, London, has obtained a quantity of amikacin suitable to serve as an international reference preparation. It learned also that a collaborative assay will be arranged by that institute when the material has been distributed into ampoules.

5. Sisomicin

The Committee was informed that, in accordance with the request made in its twenty-ninth report (I, page 12), the National Institute for
Biological Standards and Control, London, has been offered a quantity of sisomicin suitable to serve as an international reference preparation (4).

The Committee requested that institute to arrange a collaborative study.

6. Tobramycin

The Committee was informed that the collaborative assay of the proposed international reference preparation of tobramycin, referred to in its twenty-ninth report (1, page 13) has been completed and that the results are being analysed. The Committee noted that the material, which was dried over phosphorus pentoxide, is very hygroscopic and that special care is necessary when weighing it (5).

The Committee was informed also that, in accordance with the authorization in its twenty-ninth report, the National Institute for Biological Standards and Control, London, will establish the material as the international reference preparation of tobramycin and, on the basis of the results and with the agreement of the participants, will define the international unit.

7. Bleomycin complex A₂/B₂

The Committee noted that, in accordance with the request made in its twenty-ninth report (1, page 11), the National Institute for Biological Standards and Control, London, has obtained a preparation of bleomycin complex A₂/B₂ (6). The Committee was informed that, after the material had been freeze-dried in ampoules, it had been shown to be suitable. The Committee also learned that the freeze-dried material is extremely hygroscopic and that, therefore, the potency should be defined on the basis of the number of international units per ampoule. The Committee agreed that, since the two existing national standards consist predominantly of bleomycin A₂, a limited collaborative study should be arranged.

8. Bleomycin A₂

The Committee was informed that there is a need for an international reference material for bleomycin A₂—an antitumour
antibiotic preparations of which consist predominantly of one bleomycin component, but may contain small amounts of other bleomycin components.

The Committee therefore requested the National Institute for Biological Standards and Control, London, to obtain samples of bleomycin A, being used clinically, and to compare them with existing national standards.

ANTIBODIES

9. Antithyroglobulin Serum

The Committee noted that a limited international collaborative assay of the antithyroglobulin serum referred to in its twenty-first report (7, page 22), has been completed (8) and that the serum has been found to be suitable to serve as a reference preparation for the measurement of antithyroglobulin activity.

The Committee established this preparation as the International Reference Preparation of Antithyroglobulin Serum and, on the basis of the results of the collaborative study, defined the activity of the contents of each ampoule of the International Reference Preparation of Antithyroglobulin Serum as 1000 International Units of Antithyro-globulin Serum.

10. Antithyroid Microsome Serum

The Committee noted that the National Institute for Biological Standards and Control, London, has a preparation of serum containing high complement-fixing activity to thyroid microsome antigens (9). This serum has been freeze-dried and placed in ampoules. The Committee agreed that there is at present no need for an international reference preparation of antithyroid microsome serum, but requested that institute to continue to make the preparation available for research purposes.

ANTIGENS

11. Diphtheria and Tetanus Toxoids for Flocculation Tests

The Committee was informed that the request made in its twenty-second report (10, page 18)—that the State Serum Institute,
Copenhagen, should arrange a collaborative study to measure the Lf equivalents of toxoids, not by the use of reference antitoxins, but in relation to the International Standards for the Toxoids—has not been carried out because the institute has not been able to allocate the necessary resources.

The Committee learned also that some manufacturers are already using reference toxoids in their flocculation tests, and it requested WHO to collect further information on this practice.

12. *Clostridium welchii (Cl. perfringens)*
Beta and Epsilon Toxoids

The Committee noted that, in accordance with the suggestion made in its twenty-seventh report (11, page 14), the International Reference Preparation of *Clostridium welchii (Cl. perfringens)* Beta Toxoid and the International Reference Preparation of *Clostridium welchii (Cl. perfringens)* Epsilon Toxoid have been studied by the Central Veterinary Laboratory, Weybridge, for their ability to flocculate with specific antitoxins (12). It has been shown that freeze-drying the two toxoids has markedly decreased their flocculating activity, whereas their total combining power has remained constant. The Committee agreed that the flocculation test does not provide a reliable indication of the potency of these toxoids.

13. Rabies Vaccine

The Committee noted the report (13) of an international collaborative study of two preparations of rabies vaccine, prepared in human diploid cell culture, both of which are considered suitable to serve as the third international reference preparation of rabies vaccine.

The Committee decided that, instead of expressing the potency of rabies vaccine as an "antigenic value", it would be more useful for the potency to be expressed in international units.

The Committee established preparation CRV 1 as the third International Reference Preparation of Rabies Vaccine, and, on the basis of the results of the collaborative study, defined the activity of the contents of each ampoule of the third International Reference Preparation of Rabies Vaccine as 10 International Units of Rabies Vaccine.
The Committee decided that preparation CRV 2 would be useful as a working standard and requested the WHO Secretariat to define, on the basis of statistical analysis of the available data, its potency relative to the third International Reference Preparation of Rabies Vaccine.

14. Anthrax Spore Vaccine

The Committee noted that a freeze-dried preparation (14) of the 34F2 vaccine strain of *Bacillus anthracis* (15) has been used for more than eight years by several laboratories for checking the potency of seed lots for vaccine production. In view of its value for this purpose, the Committee established the preparation as the International Reference Preparation of Anthrax Spore Vaccine for use in the assay of seed lots for vaccine production. The Committee defined the activity of the contents of each ampoule of the International Reference Preparation of Anthrax Spore Vaccine as 1.0 International Unit of Anthrax Spore Vaccine.

15. BCG Vaccine

The Committee was informed that many studies have taken place since 1969, when it was considered that the International Reference Preparation of BCG Vaccine should be replaced (16). Such studies have examined the ability of various strains of BCG to protect man against the disease, but no one strain is known to be superior to the others in this respect. It has become urgent to replace the International Reference Preparation of BCG vaccine.

The Committee requested the State Serum Institute, Copenhagen, to obtain a sufficient quantity of suitable BCG vaccine and to arrange, in conjunction with WHO, an international collaborative study to determine its suitability to serve as a reference preparation.

The Committee emphasized that the proposed vaccine must meet the Requirements for Dried BCG Vaccine (17). In addition, the final ampoules of the proposed vaccine should contain the highest number of culturable particles per millilitre recommended for that vaccine strain of BCG for the immunization of adults. The vaccine selected should have the necessary long-term stability required for a reference preparation.
In case it is intended to use the proposed international reference preparation as a seed authorized by WHO, the passage history of the strain should be declared to the State Serum Institute.

16. Rinderpest Vaccine

The Committee noted that, in accordance with the request made in its twenty-second report (10, page 15), the Central Veterinary Laboratory, Weybridge, has carried out a study of the suitability of a proposed international reference preparation of rinderpest cell culture vaccine (live) prepared from the Kabete "O" strain (18).

The Committee noted also that estimates of relative virus titre of a test vaccine in terms of the proposed reference preparation have varied widely from one laboratory to another. It has not been possible to determine the reason for this, although differences in titration techniques and the difficulty of maintaining rinderpest virus in a stable state under tropical conditions may have been contributory factors.

The Committee was informed that FAO, which is responsible for standardizing this material, has decided not to establish the proposed reference preparation but has asked the Central Veterinary Laboratory to continue to make it available for comparative tests of titration techniques.

17. Purified Protein Derivative (PPD) of Bovine Tuberculin

The Committee noted the progress (19) that has been made in the collaborative study of the proposed international standard for purified protein derivative (PPD) of bovine tuberculin, requested in its twenty-seventh report (11, page 15). Assays carried out in six countries in Europe (20, page 18) have shown that the relative potencies obtained in guinea-pigs sensitized with living cultures of Mycobacterium bovis differ from those obtained when killed organisms of the same species are used for sensitizing the animals (19). The Committee noted also that the Central Veterinary Laboratory, Weybridge, is to extend the collaborative assay to include laboratories in other parts of the world. This assay will include both experimentally sensitized guinea-pigs and naturally infected tuberculous cattle, in order to investigate the correlation between biological activity in guinea-pigs and that in cattle.
18. Purified Protein Derivative (PPD) of Mallein

The Committee noted that, in accordance with the request made in its twenty-ninth report (I, page 16), the Central Veterinary Laboratory, Weybridge, is examining several preparations of mallein PPD with a view to selecting one as a proposed international standard (21). Because of the known antigenic heterogeneity of the glanders bacillus, these preparations are being examined with a view to selecting one that will adequately represent the antigenic composition of the organism. The Committee was informed that, when a suitable preparation has been selected and freeze-dried in ampoules, the Central Veterinary Laboratory will arrange an international collaborative study.

19. Brucella abortus Strain 19 Vaccine
and Brucella melitensis Strain Rev. 1 Vaccine

The Committee noted that, in accordance with the request made in its twenty-ninth report (I, page 16) the Central Veterinary Laboratory, Weybridge, has asked vaccine manufacturers, control authorities, and other experts in 36 countries whether there is a need for international standards for brucellosis vaccines (22). The replies have indicated clearly that such standards are needed.

The Committee requested the Central Veterinary Laboratory to obtain preparations of strain 19 and strain Rev. 1 vaccines and to arrange collaborative studies to determine their suitability to serve as international standards.

20. Carcinoembryonic Antigen

The Committee noted that, in accordance with the request made in its twenty-seventh report (II, page 13), the Secretariat, in conjunction with the National Institute for Biological Standards and Control, London, has enquired into the unitage of carcinoembryonic antigens in use in various countries (23). As reported in the Committee's twenty-eighth report (20, page 18), it has been ascertained that the unitage to be assigned to the proposed international reference preparation of carcinoembryonic antigen is not in conflict with the units defined by any other reference preparation of this antigen.
The Committee noted also that the National Institute for Biological Standards and Control has established the material as the International Reference Preparation of Carcinoembryonic Antigen and has defined the activity of the contents of each ampoule of the International Reference Preparation of Carcinoembryonic Antigen as 100 International Units of Carcinoembryonic Antigen.

21. Tetanus Toxin

The Committee noted that the National Institute for Biological Standards and Control, London, has obtained samples of tetanus toxin and antitoxin from eight laboratories (1, page 15; 24). By means of a paralytic dose end-point, the Lp/10 dose of each toxin has been estimated in terms of the International Standard for Tetanus Antitoxin. The Lp/10 doses so determined are used to compare the neutralizing power of the eight antitoxins with that of the International Standard for Tetanus Antitoxin. The Committee noted also that the pattern of these values is irregular and that more work is needed before an international collaborative assay can be arranged.

22. Pertussis Vaccine

The Committee noted that the international collaborative assay of the proposed second international standard for pertussis vaccine has been completed (25). Results from the ten participating laboratories, which have confirmed that the proposed standard is a suitable preparation, are being analysed.

The Committee authorized the National Institute for Biological Standards and Control, London, to establish the preparation as the second international standard for pertussis vaccine and, on the basis of the results and with the agreement of the participants, to define the international unit.

23. Yellow Fever Vaccine

The Committee noted the results of the international collaborative study, referred to in its twenty-fifth report (26, page 10), in which several yellow fever vaccines were tested. The virus titres obtained by
the intracerebral inoculation of mice were compared with those obtained by means of a cell culture assay technique (27). The latter technique provides more uniform results and the inclusion of a common reference preparation gives better agreement between laboratories. Such a reference preparation would be of value in the assay of the virus concentration of yellow fever vaccines, but the Committee was informed that not enough of the material used in the study is available to provide an international reference preparation.

The Committee therefore requested the National Institute for Biological Standards and Control, London, to obtain a quantity of yellow fever vaccine suitable to serve as an international reference preparation, and to arrange an international collaborative assay to determine its acceptability and estimate its live virus content.

24. Diphtheria Toxoid, Adsorbed

The Committee noted that, in accordance with the request made in its twenty-seventh report (II, page 15), the State Serum Institute, Copenhagen, arranged a collaborative assay of the two preparations of adsorbed diphtheria toxoid, which are being considered to replace the International Standard for Diphtheria Toxoid, Adsorbed (28). Both preparations have been found to be acceptable, but, since one (DIXA-50) contains a stabilizer that is nonantigenic, this preparation is to be preferred.

The Committee therefore established preparation DIXA-50 as the second International Standard for Diphtheria Toxoid, Adsorbed and, on the basis of the results, defined the activity of the contents of each ampoule of the second International Standard for Diphtheria Toxoid, Adsorbed, as 132 International Units of Diphtheria Toxoid, Adsorbed.

BLOOD PRODUCTS AND RELATED SUBSTANCES

25. Anti-D Immunoglobulin

The Committee noted that the activity of the International Reference Preparation of Anti-D Immunoglobulin, Human, was
inadvertently not calibrated in terms of the first International Standard for Anti-Rho (anti-D) Incomplete Blood Typing Serum, Human, in the original collaborative assay (29). Consequently the activity of 150 International Units originally stated (20, page 11) to be contained in each ampoule of the International Reference Preparation is incorrect, although the stated content of 60 μg of anti-D immunoglobulin in each ampoule is correct.

The Committee noted the results of a more recent collaborative assay in which the International Reference Preparation of Anti-D Immunoglobulin, Human, was calibrated in terms of the first International Standard for Anti-Rho (anti-D) Incomplete Blood Typing Serum (30). On the basis of the results of the collaborative assay, the Committee defined the activity of the contents of each ampoule of the International Reference Preparation of Anti-D Immunoglobulin, Human, as 300 International Units of Anti-D Immunoglobulin, Human.

26. Antithrombin III

The Committee noted the results of a collaborative assay of antithrombin III organized by the National Institute for Biological Standards and Control, London, which included two preparations of purified human antithrombin III and a pool of freeze-dried normal plasma (31). Less variation of potency estimates was obtained when the freeze-dried normal plasma preparation was used as the reference material.

The Committee therefore established the freeze-dried normal plasma (NIHSC 72/1) as the International Reference Preparation of Antithrombin III and, on the basis of the results of the collaborative assay, defined the activity of the contents of each ampoule of the International Reference Preparation of Antithrombin III as 0.9 International Unit of Antithrombin III.

27. Anti-A, Anti-B, Anti-(A + B), Anti-C, and Anti-E
Blood Typing Sera

The Committee noted that a pool of suitable anti-B blood typing sera has been freeze-dried in ampoules and that an international collaborative study has been completed by the Central Laboratory of
the Netherlands Red Cross Blood Transfusion Service, Amsterdam, in which the activity of the proposed replacement preparation has been compared with that of the International Standard for Anti-B Blood Typing Serum (32).

The Committee authorized that laboratory to establish the preparation as the second international standard for anti-B blood typing serum, and, on the basis of the results and with the agreement of the participants, to define its potency in international units relative to the first International Standard for Anti-B Blood Typing Serum, Human.

The Committee was informed also that the collection of anti-A, anti-(A+B), anti-C, and anti-E blood typing sera by that laboratory is in progress.

28. Blood Group Substances A and B

The Committee noted that the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, has requested blood group substances A and B, both animal and synthetic, from several sources and that some preparations are being examined (33).

The Committee requested that laboratory to arrange an international collaborative study to determine the suitability of such preparations.

29. Anti-Hepatitis A Immunoglobulin

The Committee noted the progress made by the Bureau of Biologics, Food and Drug Administration, Bethesda, MD, USA, in obtaining suitable material for an international reference preparation of anti-hepatitis A immunoglobulin and in arranging, as requested in its twenty-ninth report (1, page 18), an international collaborative study (34).

The Committee noted also that the study has been designed to compare the proposed international reference preparation with the anti-HAV panel of anti-hepatitis A immune serum globulins of the Bureau of Biologics.
30. Thromboplastin (Bovine, Combined) and Thromboplastin (Rabbit, Plain)

The Committee noted that, to enable more precise calibration of thromboplastins to be done, it is necessary to have a thromboplastin reference preparation of each type (human, bovine, and rabbit) (35). At its twenty-eighth meeting the Committee established only the International Reference Preparation of Thromboplastin (Human, Combined) (20, page 14).

The Committee noted also that, in order to make progress in the formulation of requirements, the Director-General had established the International Reference Preparation of Thromboplastin (Bovine, Combined) with an international calibration constant of 1.0 and the International Reference Preparation of Thromboplastin (Rabbit, Plain) with an international calibration constant of 0.6. Both these preparations had been included in international collaborative studies in which they were related to the International Reference Preparation of Thromboplastin (Human, Combined) and the international calibration constants had been determined on the basis of the results.

ENDOCRINOLOGICAL AND RELATED SUBSTANCES

31. Oxytocin for Bioassay

The Committee noted the results (36) of the international collaborative assay of the proposed fourth international standard for oxytocin, for bioassay, mentioned in its twenty-ninth report (1, page 22).

The Committee established the preparation of purified synthetic oxytocin studied as the fourth International Standard for Oxytocin for Bioassay, and, on the basis of the results of the collaborative assay, defined the activity of the contents of each ampoule of the fourth International Standard for Oxytocin, for Bioassay, as 12.5 International Units of Oxytocin for Bioassay.

32. Arginine Vasopressin for Bioassay

The Committee noted the report of the collaborative assay (37) mentioned in its twenty-ninth report (1, page 22). The Committee
established the preparation of arginine vasopressin studied as the International Standard for Arginine Vasopressin, for Bio assay, and, on the basis of the results, it defined the activity of the contents of each ampoule of the International Standard for Arginine Vasopressin, for Bio assay, as 8.2 International Units of Arginine Vasopressin, for Bio assay.

33. Oxytocin and Vasopressin, Bovine, for Bio assay

The Committee noted that the fourth International Standard for Oxytocin, for Bio assay, and the first International Standard for Arginine Vasopressin, for Bio assay, have now been established and that these two standards, which consist of highly purified peptides, replace the third International Standard for Oxytocin and Vasopressin, Bovine, for Bio assay, which consisted of dried acetone-extracted posterior pituitary powder.\(^1\)

The Committee therefore discontinued the third International Standard for Oxytocin and Vasopressin, Bovine, for Bio assay.

34. Lysine Vasopressin

The Committee noted the results (38) of the collaborative assay of the International Standard for Lysine Vasopressin referred to in its twenty-ninth report (I, page 22). On the basis of those results, the Committee defined the activity of the contents of each ampoule of the International Standard for Lysine Vasopressin as 7.7 International Units of Lysine Vasopressin.

35. Desmopressin

The Committee noted that, in accordance with the request made in its twenty-ninth report (I, page 23), the National Institute for Biological Standards and Control, London, has obtained a quantity of purified desmopressin to serve as an international reference material, and has freeze-dried it in ampoules (39). The Committee noted also that an international collaborative study is in progress.

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\(^1\) See sections 31 and 32 above.
36. Prolactin, Human, for Immunoassay

The Committee noted that, in accordance with the authorization given in its twenty-ninth report (I, page 20), the National Institute for Biological Standards and Control, London, has established the International Reference Preparation of Prolactin, Human, for Immunoassay (40), and, on the basis of the results of the international collaborative assay and with the agreement of the participants, has defined the activity of the contents of each ampoule of the International Reference Preparation of Prolactin, Human, for Immunoassay, as 0.65 International Unit of Prolactin, Human, for Immunoassay.

37. Human Corticotrophin (ACTH) for Immunoassay

The Committee noted that, in accordance with the request made in its twenty-sixth report (41, page 20), the National Institute for Biological Standards and Control, London, has obtained a highly purified preparation of human corticotrophin (ACTH) and has studied its activity (42). The Committee noted also that there are too few ampoules of this preparation to make it possible to establish it as an international reference material, and that a further, larger quantity of similar material is being obtained. The Committee requested the Institute to arrange an international collaborative study and, in the meantime, to make the material previously studied available to research workers.

38. Thyroid-Stimulating Hormone, Human, for Immunoassay

The Committee noted that, in accordance with the request made in its twenty-sixth report (41, page 14), the National Institute for Biological Standards and Control, London, has obtained two preparations of highly purified thyroid-stimulating hormone, one of which in due course will replace the International Reference Preparation of Thyroid-Stimulating Hormone, Human, for Immunoassay (43). The Committee noted also that work is in progress to determine the purity and suitability of the preparation most appropriate for an international reference preparation. The Committee was informed that there is an extensive international need for a working standard of this
preparation, and requested the Institute to consider the possibility of preparing such a working standard in addition to the international reference preparation. This would be in accordance with the general recommendations made in the Guidelines for the Preparation and Establishment of Reference Materials and Reference Reagents for Biological Substances (I, page 129).

39. Pituitary LH(ICS), Human, for Immunoassay

The Committee noted that six preparations have been offered to the National Institute for Biological Standards and Control, London, as materials proposed for the replacement of the International Reference Preparation of Pituitary LH(ICS), Human, for Immunoassay (44).

The Committee noted also that extensive studies are in progress to determine the degree of contamination of these preparations with other pituitary glycoprotein hormones and their subunits before selecting the most appropriate preparation.

40. Luteinizing Hormone, Pituitary, Alpha and Beta Subunits, Human

The Committee noted that, in accordance with the request made in its twenty-sixth report (41, page 11), the National Institute for Biological Standards and Control, London, has obtained quantities of the highly purified alpha and beta subunits of human pituitary luteinizing hormone (45). The Committee was informed that these materials have been freeze-dried in ampoules and are being studied for their suitability to serve as international reference materials.

41. Follicle-Stimulating Hormone, Pituitary, Human, for Immunoassay

The Committee noted that, in accordance with the request made in its twenty-sixth report (41, page 14), the National Institute for Biological Standards and Control, London, has obtained a highly purified preparation of human pituitary follicle-stimulating hormone,
which has been freeze-dried in ampoules (46). The Committee noted also that an international collaborative study of the preparation is being arranged.

42. Chorionic Gonadotrophin, Human, for Immunoassay

The Committee noted that, in accordance with the request made in its twenty-sixth report (41, page 12), the International Reference Preparation of Chorionic Gonadotrophin, Human, for Immunoassay, has been calibrated by bioassay against the second International Standard for Chorionic Gonadotrophin, Human, for Bioassay, and that a proposal for a unitage (650 per ampoule) to be assigned to it has been made on the basis of the results (47).

The two preparations have also been compared by means of various immunoassay systems. The results (48) of these assays were heterogeneous, as is to be expected when comparing a relatively impure preparation (the second International Standard for Chorionic Gonadotrophin, Human, for Bioassay) with a highly purified preparation (the International Reference Preparation of Chorionic Gonadotrophin, Human, for Immunoassay).

The Committee noted also that one of the eleven participants in the collaborative assay objected to the allocation of the unitage on the basis of bioassays because it would lead to changes in the numerical values used to specify the sensitivity and ranges of normal values obtained with pregnancy test kits.

Nevertheless, in accordance with the recommendation made in its twenty-first report (7, page 8), the Committee defined the activity of the contents of each ampoule of the International Reference Preparation of Chorionic Gonadotrophin, Human, for Immunoassay, as 650 International Units of Chorionic Gonadotrophin, Human, for Immunoassay.

The sensitivity of pregnancy test kits involving immunoassay or analogous (binding) procedures, which has been specified hitherto in terms of International Units of Chorionic Gonadotrophin, Human, for Bioassay, should now be specified in terms of International Units of Chorionic Gonadotrophin, Human, for Immunoassay.

In order to diminish confusion in the transition to the use of the new International Unit, the Committee recommended that for an interim period the sensitivity and ranges of normal values of such kits should be stated in terms of International Units of Chorionic
Gonadotrophin, Human, for Bioassay, as well as in International Units of Chorionic Gonadotrophin, Human, for Immunoassay. The Committee emphasized that this step will not necessarily involve a change in the reagents themselves.

43. Human Insulin C-Peptide for Immunoassay

The Committee noted that, in accordance with the request made in its twenty-sixth report (41, page 17), the National Institute for Biological Standards and Control, London, has confirmed that a reference preparation of human insulin C-peptide may serve a useful purpose (49). It was noted, however, that insufficient natural (or even synthetic) human C-peptide is available, the only material obtainable being the synthetic analogue, 64 formyllysine C-peptide. The Institute has obtained a sample of this material and freeze-dried it in capillary ampoules.

The Committee noted also the results of the international collaborative assay arranged by the National Institute for Biological Standards and Control, which have shown that estimates of C-peptide in plasma samples differ according to the specificity of the assay system used. The Committee agreed, therefore, that the synthetic analogue cannot serve as an international reference preparation for the assay of natural C-peptide. Nevertheless, the Committee agreed that the preparation would be useful for research purposes and noted that the Institute will make it available.

44. Calcitonin, Human, for Bioassay

The Committee noted that, in accordance with the request made in its twenty-sixth report (41, page 8), the National Institute for Biological Standards and Control, London, has confirmed that an international reference preparation is necessary for the control of preparations of synthetic human calcitonin (50).

The Committee noted also that the Institute has obtained a quantity of synthetic calcitonin of the amino acid sequence found in calcitonin from human tumours. The materials have been freeze-dried in ampoules and have been included in an international collaborative study together with other synthetic preparations of this hormone. The results of the study have shown that the material is stable and suitable to serve as an international reference material for bioassay (50).
The Committee therefore established the preparation as the International Reference Preparation of Calcitonin, Human, for Bioassay, and defined the activity of the contents of each ampoule of the International Reference Preparation of Calcitonin, Human, for Bioassay, as 1.0 International Unit of Calcitonin, Human, for Bioassay.

45. Gonadotrophin-Releasing Hormone (Gonadorelin)

The Committee noted that, in accordance with the request made in its twenty-sixth report (41, page 19), the National Institute for Biological Standards and Control, London, has obtained a quantity of synthetic gonadorelin (a decapeptide of the amino acid sequence determined for sheep), which causes the release of luteinizing hormone and follicle-stimulating hormone from the pituitary.

The Committee noted also that the preparation has been freeze-dried in ampoules and that an international collaborative study is in progress (37).

46. Kininogenase (Kininogenin)

The Committee noted that, in accordance with the request made in its twenty-ninth report (1, page 23), the National Institute for Biological Standards and Control, London (52), has obtained and freeze-dried in ampoules a highly purified preparation of porcine pancreatic kininogenase (kininogenin (EC 3.4.21.8)). The Committee noted also that preliminary studies have shown the preparation to be stable and that an international collaborative study is being arranged.

REAGENTS

47. Hepatitis B Serum Panels

The Committee noted that the WHO Collaborating Centre for Reference and Research on Viral Hepatitis, Center for Disease Control, Phoenix, AZ, USA, has selected four groups of sera, referred to as “serum panels”, for the detection of HBsAg, anti-HBs, subtypes of HBsAg, and HBeAg/anti-HBe (53), respectively. The Committee
noted also the results of the tests of the stability and specificity of the serum panels.

The Committee recommended that these panels should be designated as WHO International Serum Panels and that they should be maintained and distributed by the WHO Collaborating Centre for Reference and Research on Viral Hepatitis.

48. Leptospira Reference Sera

The Committee noted that, in accordance with the request made in its twenty-ninth report (1, page 24), the Central Veterinary Laboratory, Weybridge, has enquired into the possible need for additional reference reagents for the identification of strains of leptospires (54). This enquiry has shown that most laboratories would like additional reference sera but that there is no general agreement on the criteria that such reagents should meet.

The Committee was informed, however, that the Subcommittee on the Taxonomy of Leptospira of the International Association of Microbiological Societies decided recently to divide the genus into two species (L. interrogans and L. biflexa) and has agreed on standard methods for preparing reference sera and testing them for specificity and potency. The standard methods would involve the use of more highly specific sera that would render the existing international reference reagents inappropriate. The Committee was informed also that the subcommittee is investigating the use of group sera for initial screening purposes.

The Committee noted the views of the subcommittee but agreed that, until such time as the more specific sera are available and have been shown to be suitable for international use, the international reference reagents of leptospira sera should continue to be made available. The Committee requested the Central Veterinary Laboratory to keep the situation under review.

49. Adenovirus Antisera (Equine) Types 25 to 33 inclusive

The Committee noted the results of tests on antisera prepared in horses by the Center for Disease Control, Atlanta, GA, USA, against adenoviruses types 25 to 33 inclusive (55). These antisera have been prepared in a manner similar to that used for preparing the antisera
to adenoviruses 1–24, most of which have already been established as International Reference Reagents.

The tests showed that the equine antisera to types 25–33 were stable and had minimal heterologous serum neutralizing titres, except for type 29 antiserum with type 15 virus.

These two viruses, however, could be differentiated easily by the haemagglutination inhibition (HI) test. All homologous HI titres were adequate except for antiserum to type 28, which was marginal. It was considered by the five participants in the study that the results were satisfactory.

The Committee, therefore, established the freeze-dried sera as the International Reference Reagents of Adenovirus Antisera, Equine, for Adenovirus Types 25 to 33 inclusive.

**MISCELLANEOUS**

**50. Interferons**

The Committee noted (56, 57) the resurgence of interest in interferons, several preparations of which were considered in its nineteenth report (38, page 25) and twenty-first report (7, page 23). In order that progress may be made in the clinical application of human interferon as well as in research into animal models of interferon activity, the Committee established:

1. preparation 69/19 (MRC Research Standard B) as the International Reference Preparation of Interferon, Human Leukocyte, and defined the activity of the contents of each ampoule of the International Reference Preparation of Interferon, Human Leukocyte, as 5000 International Units of Interferon, Human Leukocyte;

2. the NIH preparation G 023-902-527 as the International Reference Preparation of Interferon, Human Fibroblast, and defined the activity of the contents of each ampoule of the International Reference Preparation of Interferon, Human Fibroblast, as 10,000 International Units of Interferon, Human Fibroblast;

3. the NIH preparation G 002-904-511 as the International Reference Preparation of Interferon, Mouse, and defined the activity of the contents of each ampoule of the International Reference Preparation of Interferon, Mouse, as 12,000 International Units of Interferon, Mouse;
(4) the NIH preparation G 019-902.528 as the International Reference Preparation of Interferon, Rabbit, and defined the activity of the contents of each ampoule of the International Reference Preparation of Interferon, Rabbit, as 10,000 International Units of Interferon, Rabbit;

(5) preparation 67/18 (proposed replacement British Research Standard B) as the International Reference Preparation of Interferon, Chick, and defined the activity of the contents of each ampoule of the International Reference Preparation of Interferon, Chick, as 80 International Units of Interferon, Chick.

51. Pyrogens

The Committee considered a report (59) on present methods of testing for pyrogens. Although much research has gone into the possibility of replacing the rabbit test by the Limulus amoebocyte lysate test — a rapid and simple procedure — the Limulus test has the disadvantage of being specific for endotoxins only from Gram-negative organisms. The Limulus test is to be preferred for certain purposes and preparations, but it does not appear that it could replace the rabbit test in the detection of all materials giving rise to a pyrogenic reaction. The Committee requested the National Institute for Biological Standards and Control, London, to assess the need for additional reference preparations of other pyrogens and Limulus lysates for the purpose of standardizing the sensitivity of batches of Limulus reagents.

The Committee agreed with the proposal of the Institute to make small quantities of the International Reference Preparation of Pyrogen widely available.

**REQUIREMENTS FOR BIOLOGICAL SUBSTANCES**

52. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines

The Committee noted that, in accordance with the request made in its twenty-ninth report (I, page 27), WHO has revised (60) the
Requirements for Diphtheria Toxoid and Tetanus Toxoid (61) and the Requirements for Pertussis Vaccine (62).

Since the manufacturers of these vaccines usually produce all three vaccines, and since the three are generally given together as a mixture, it was considered appropriate to reformulate the revised requirements in a single document. The revised requirements, however, consider each vaccine separately so that they would be appropriate for the manufacture and control of the vaccines individually.

The Committee noted also that the requirements for each vaccine are followed by a suggested protocol for use by manufacturers in reporting their results to control authorities. Such protocols would be useful also for sending to countries that import vaccines but have no control laboratories.

After making some minor amendments, the Committee adopted the Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Revised 1978) and agreed that they should be annexed to this report (Annex I).

53. Proposed Guidelines for Antitumour Antibiotics

The Committee noted that, in accordance with the request made in its twenty-ninth report (I, page 12), WHO has formulated guidelines for the control of antitumour antibiotics, but that it was not possible to assemble all the technical data for the characterization of each antibiotic in time to complete the document for presentation to this meeting of the Committee (63).

The Committee agreed that, in view of the importance of the guidelines, they should be distributed as soon as agreement has been reached on their formulation, and that their formal acceptance should be considered at the next meeting of the Committee.

54. Requirements for Rabies Vaccine (Human and Veterinary)

The Committee noted that, in accordance with the request made in its twenty-ninth report (I, page 26), WHO has considered the formulation of requirements for rabies vaccine for veterinary use (64). Furthermore, WHO has been considering the possibility of coupling the formulation of requirements for rabies vaccine for veterinary use with the revision of the Requirements for Rabies
Vaccine for Human Use (26, Annex 3, page 22) and having the two requirements in a single document. The Committee agreed that such a possibility should be explored, not only because of the need to include the production of rabies vaccine in human diploid cells for human use, but also because of the similarity of the two vaccines. The Committee requested WHO to look into this possibility and to formulate the necessary requirements.

55. Requirements for Thromboplastins

The Committee noted that, in accordance with the recommendation made in its twenty-eighth report (29, page 51), WHO has formulated, for national authorities, guidelines for implementing the international calibration scale at the national level (65). These guidelines have been considered by a number of experts and are now ready for distribution to the Expert Advisory Panel on Biological Standardization.

The Committee agreed that, in view of the importance of the guidelines, they should be distributed as soon as agreement has been reached on their formulation, and that their formal acceptance should be considered at the next meeting of the Committee.

56. Requirements for Dried BCG Vaccine

The Committee noted that, in accordance with the request made in its twenty-ninth report (1, page 27), WHO has revised the Requirements for Dried BCG Vaccine (17). The Committee noted also that BCG vaccines are now required to satisfy tests for stability and the number of culturable particles.

The Committee noted, furthermore, that there is a need for a replacement of the International Reference Preparation of BCG vaccine. The preparation established in 1965 is no longer representative of the strains from which the majority of BCG vaccines are now produced. The Committee requested the State Serum Institute, Copenhagen, to obtain a replacement reference preparation and to arrange an international collaborative assay to determine its suitability and measure the number of culturable particles.

After making some minor amendments, the Committee adopted the Requirements for Dried BCG Vaccine (Revised 1978) and agreed that they should be annexed to this report (Annex 2).
57. Requirements for Influenza Vaccine (Inactivated) and for Influenza Vaccine (Live)

The Committee noted that, in accordance with the suggestion made in its twenty-ninth report (I, page 25), WHO had revised the Requirements for Influenza Vaccine (Inactivated) (66), and that WHO considered it appropriate to formulate requirements for live influenza vaccine (66).

The Committee noted also that the content of haemagglutinin — the antigen recognized as that responsible for the development of protection against the disease — can now be measured reliably by means of an immunodiffusion technique.

In accordance with the decision of the Committee (I, page 14), the reference materials for such controls are referred to as WHO Influenza Virus Reference Haemagglutinin, with the year of production given in brackets.

As far as live vaccine is concerned, it was recognized as critical that technology should be developed to ensure that a stable attenuated strain is used.

After making a few minor amendments, the Committee adopted the Requirements for Influenza Vaccine (Inactivated) (Revised 1978) and the Requirements for Influenza Vaccine (Live), and agreed that they should be annexed to this report (Annex 3).

ACKNOWLEDGEMENTS

The Committee thanks the following members of the WHO Secretariat for their special contributions to its deliberations: Dr K. Bögel, Veterinary Public Health; Dr K. Schlenzka, Senior Legal Officer, Legal Division; and Dr W. Wieniawski, Chief Pharmaceutical Officer, Pharmaceuticals.
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Annex 1

REQUIREMENTS FOR DIPHTHERIA TOXOID, PERTUSSIS VACCINE, TETANUS TOXOID, AND COMBINED VACCINES

(Requirements for Biological Substances Nos. 8 and 10)
(Revised 1978)

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INTRODUCTION

Requirements for Pertussis Vaccine (1, Annex I) were formulated in 1963 and for Diphtheria and Tetanus Toxoids (2) in 1964. Since then there have been several developments in the production and
testing of these products. Such changes are concerned mainly with production methods and potency assays, but they are of sufficient significance to justify a revision of the requirements for each of the vaccines.

As the vaccine used most commonly is diphtheria, pertussis, tetanus (DPT) it has been decided to publish the revised requirements for all three vaccines in a single document. Although the vaccines are used in the combined form and usually contain an adjuvant, the manufacturing requirements are dealt with in separate sections individually. A fourth section has been included because there are some special tests applicable to the vaccines only when in a combined formulation. Protocols for reporting the data for each vaccine as well as for the combined vaccines have also been included.

The following revised international requirements for DPT were drafted by a group of WHO consultants and staff members (listed on page 113). Account has been taken of the regulations for DPT vaccines that are in force in a number of countries. In addition, opinions and data have been received from a number of experts, whose assistance is gratefully acknowledged (see page 114).

REQUIREMENTS FOR DIPHTHERIA TOXOID

GENERAL CONSIDERATIONS

Diphtheria toxoid was one of the earliest vaccines available for protection against a bacterial disease and its use, when of proved efficacy in immunization schedules known to give good antitoxin responses, has markedly reduced the incidence of the disease.

The early developments leading to the formulation of the first Requirements for Diphtheria Toxoid are described in detail in the Introduction and General Considerations to Requirements for Biological Substances No. 10 (2, Annex 1). The purpose of the present General Considerations is to draw attention to the significant developments that have taken place since the first requirements were formulated.

One of the most important considerations is the agreement reached on the formulation of requirements for the assay of potency. In 1964, almost all countries adopted their own requirements and there was little uniformity between them. There is greater uniformity of opinion concerning the potency requirements for diphtheria
toxoid needed to ensure adequate protection against the disease. Accordingly, it has been possible to include a requirement that involves the immunization of guinea-pigs and subsequent challenge either by a lethal challenge dose of toxin or by an intradermal challenge using graded doses of the challenge toxin. The lethal challenge test gives an indication that the immunized animals are protected against a single challenge dose of toxin, whereas the intradermal challenge test, by virtue of its graded doses of challenge, gives a measure of how much protection the immunized animals have against a particular dose of toxin. Clearly, the latter test gives more information than the former. Such a test is recommended, but the decision concerning the details of the particular test to adopt is left to the national control authority. It is important to include in each test a reference preparation that has been calibrated in International Units. In this way the potency of the vaccine may also be quoted in IU.

The strain of Corynebacterium diphtheriae (Parke Williams 8) from which the toxoid is made has been shown to be satisfactory for producing potent diphtheria vaccines. There seems to be no purpose in suggesting a change of strain. The main aim of good vaccine production is to obtain the greatest quantity of toxin during the growth phase of the organisms and, thereafter, to convert the toxin into toxoid by the most efficient method.

The WHO Expert Committee on Biological Standardization, recognizing the limitations of the various methods in common use, established, in 1951, the International Standard for Diphtheria Toxoid, Plain, in order that toxoids might be assayed by comparative measurement with this toxoid standard. Furthermore, the Expert Committee established, in 1955, the International Standard for Diphtheria Toxoid, Adsorbed, because the log dose/response lines of plain and adsorbed toxoids are not parallel. Thus a statistically valid comparison of an adsorbed toxoid with a plain standard was not possible. The International Standard for Diphtheria Toxoid, Plain, was replaced in 1975, the International Standard for Diphtheria Toxoid, Adsorbed, in 1978.

Although there are few data to permit the correlation of a potency level in a biological assay with protection in man, and even fewer for correlation between the potency level and the duration of immunity, sufficient evidence is available to assign a minimum potency level above which a vaccine may be considered to be of acceptable potency. Such levels have been incorporated into the present Requirements.
In the present International Requirements for Diphtheria Toxoid, purification of the product is required. Diphtheria toxoid in the unpurified form is liable to give severe vaccination reactions in man and much work has been done in developing purified material to avoid such reactions. Even with purified products, however, untoward reactions may occur in adults. In view of the risk of reversal to toxicity when a toxin is detoxified after purification, the requirements have been formulated to exclude this risk. There is evidence that purification, although enabling more concentrated preparations to be used, may sometimes reduce the immunizing activity of diphtheria toxoid, probably on account of the removal of substances having an adjuvant effect. Such purified products, if intended for primary immunization, should be combined either with pertussis vaccine containing whole organisms or with a mineral adjuvant (or both), although they may be used uncombined for reinforcing immunity.¹

Each of the following sections constitutes a recommendation. The parts of each section that are printed in type of normal size have been written in the form of requirements, so that, if a health administration so desires, these parts as they appear may be used as definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning diphtheria vaccine, it is recommended that a clause be included permitting modifications of the manufacturing requirements on condition that such modified requirements ensure that the degree of safety and the potency of the toxoids are at least equal to those provided by the requirements formulated below. The World Health Organization should then be informed of the action taken.

The terms “national control authority” and “national control laboratory”, as used in these requirements, always refer to the country in which the toxoid is manufactured.

¹ Primary immunization refers to the initial course of injections, usually consisting of 2 (or 3) injections at an interval of 4–6 weeks, followed by a further injection 7–12 months later. Reinforcing immunity, which is sometimes called “boosting” immunity, refers to subsequent single injections, usually given a number of years later.
PART A. MANUFACTURING REQUIREMENTS

A.1. DEFINITION

A.1.1 International name and proper name

The international name shall be "Vaccinum Diphtheriae". The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to toxoids that satisfy the requirements formulated below.

A.1.2 Descriptive definition

Vaccinum Diphtheriae is a preparation of diphtheria toxoid prepared by treating diphtheria toxin by chemical means to render it non-toxic without destroying its immunogenic potency. The preparation shall satisfy the requirements formulated below.

The most common method of preparing toxoids from toxins is by means of formaldehyde.

A.1.3 International Standards, International Reference Preparations, and International Units

The International Standard for Diphtheria Antitoxin (established in 1934) is stored in ampoules containing dried hyperimmune horse serum, and is dispensed as a solution of the serum in saline containing 660 ml of glycerol per litre in a concentration of 10 International Units per ml. The International Unit is defined as the activity contained in 0.0628 mg of the dry material in the stock ampoules of the International Standard.

The Fifth International Reference Preparation of Diphtheria Antitoxin for Flocculation Test (established in 1971) consists of a hyperimmune horse serum and is dispensed in ampoules containing 1800 IU equivalents per ampoule and freeze-dried.

The Second International Standard for Diphtheria Toxoid, Plain (established in 1975), is dispensed in ampoules containing 200 IU per ampoule and freeze-dried.

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2 The international reference materials should be used by reconstituting the whole contents of the ampoules in a given volume and using the reconstituted material with a calculated number of units per millilitre, depending on the volume of fluid used and the declared content of each ampoule.
DIPHTHERIA

The Second International Standard for Diphtheria Toxoid, Adsorbed (established in 1978), is dispensed in ampoules containing 122 IU and freeze-dried.

The International Standard for Schick Test Toxin (Diphtheria) (established in 1954) is dispensed in ampoules containing 900 IU and freeze-dried.

The above-mentioned Standards and Reference Preparation are in the custody of the International Laboratory for Biological Standards, State Serum Institute, Copenhagen. Samples are distributed free of charge, on request, to national control laboratories. The International Standards and International Reference Preparations are intended for the calibration of national standards and reference preparations for use in the manufacture and laboratory control of diphtheria antitoxin, vaccines, and Schick test toxin.

A.1.4 Terminology

Single harvest. The toxic filtrate obtained from one batch of cultures inoculated, harvested, and processed together.

Bulk purified toxoid. The processed purified material prepared from either a single harvest or a pool of a number of single harvests. It is the parent material from which the final bulk is prepared.

Final bulk. The homogeneous final toxoid present in a single container from which the final containers are filled either directly or through one or more intermediate containers.

Final lot. A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling. A final lot must, therefore, have been filled from a single container in one working session.

A.2 GENERAL MANUFACTURING REQUIREMENTS

The general requirements for manufacturing establishments, contained in revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, Annex 1) shall apply to establishments manufacturing diphtheria toxoid, with the addition of the following:

Written descriptions of procedures for the preparation of diphtheria toxoid adopted by a manufacturer shall be submitted for approval to the national control authority. Proposals for modifications shall also be submitted for approval to the national control authority before their implementation.
A.3 PRODUCTION CONTROL

A.3.1 Control of source materials

A.3.1.1 Strains of Corynebacterium diphtheriae

Strains of *C. diphtheriae* used in preparing diphtheria toxoid shall be identified by a record of their history and of all tests made periodically for verification of strain characters. The strain shall be maintained as a freeze-dried culture.

A highly toxigenic strain of *C. diphtheriae* should be used. A strain that has proved satisfactory in many laboratories is the Parke Williams 8 strain.

A.3.1.2 Seed lot system

The production of diphtheria toxin shall be based on a seed lot system. Cultures of the working seed shall have the same characteristics as cultures of the strains from which the parent seed lot was derived. The preparation of seed lots shall be in compliance with the requirements of Part A, Section A.3.2.

A.3.1.3 Culture medium for production of toxin

The medium shall be free from ingredients that will be present in the final product and that are known to cause toxic or allergic reactions in man.

Since some medium components may be present in the finished product, it is particularly important to ensure that the final product is free from substances that are likely to cause toxic or allergic reactions in man. If the medium is prepared from protein digest, e.g., casein hydrolysate or digested muscle, precautions should be taken to ensure that digestion has proceeded sufficiently to free the medium from such substances. Neither mammalian protein nor human blood group substances should be present in the final vaccine; the method of detecting these substances should be approved by the national control authority.
A.3.2 Production precautions

The general production precautions, as formulated in Part A, section 3, of Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 15) shall apply to the manufacture of diphtheria toxoid.

Suitable methods for the production of diphtheria toxoid are given in the Manual for the production and control of vaccines: diphtheria toxoid (4).

A.3.3 Control of single harvests

The production shall be shown to be consistent by observing the growth, pH, and rate of toxin production.

Any culture showing anomalous growth characteristics should be investigated and shown to be satisfactory before being accepted as a single harvest.

A.3.3.1 Control of bacterial purity

Samples of the cultures used for preparing a single harvest of toxoid shall be tested for bacterial purity by microscopic examination of stained smears and by inoculation into appropriate culture media. Single harvests shall not be used for preparing bulk material if contamination has occurred at any stage in their production.

A.3.3.2 Filtration

After sampling for the control of purity, the culture shall be filtered with a filter capable of producing a clear filtrate.

The cultures should be filtered as soon as possible after the end of their incubation period. To facilitate filtration, the cultures may be centrifuged and/or a filter aid may be added beforehand.

In some countries any filter shedding fibres may not be used.

The cultures may be treated with a preservative before filtration. On no account should phenol be used.

A.3.3.3 Determination of the concentration of toxin (Lf)

The toxic filtrate shall be tested by flocculation in comparison with a reference material calibrated against the International
Reference Preparation of Diphtheria Antitoxin for Flocculation Test or an equivalent reference preparation approved by the national control authority.

A suitable method is described in the Manual (4, Appendix D.7).

It is preferable for toxic filtrates used in preparing purified toxoid to contain at least 50 Lf/ml.

This test is not an absolute measure of potency, but it is a good guide to the consistency of production.

A.3.3.4 Detoxification and purification of toxin

The purification process may either precede or follow detoxification, and the method used shall be approved by the national control authority. The agent used (formaldehyde or glutaraldehyde) shall fulfil the requirements of the International Pharmacopoeia (5, page 228) or other specifications approved by the national control authority.

Any free detoxifying agent shall be removed or neutralized after the completion of detoxification. The method used shall be approved by the national control authority.

Care should be taken to ensure that reversal to toxin does not take place on storage. Methods for the detection of reversal are suggested in the Manual (4, Appendix D.9).

In some countries the following test is applied to check the possibility of toxicity reversal. One sample is diluted to 200 Lf/ml (with 0.0167 mol/l phosphate-buffered saline), and another sample to a concentration equivalent to that of the final bulk. The latter sample is kept standing at 37°C for 20 days. Of each sample 5 ml are injected subcutaneously into at least 5 guinea-pigs, which are then observed for 6 weeks (as described in Part A, Section A.3.4.3).

The method of purification shall be such that no substances are incorporated into the final product that are likely to cause untoward reactions in man.

A.3.4 Control of bulk purified toxoid

A.3.4.1 Preparation

The bulk purified toxoid shall be prepared from either a single harvest or a pool of a number of single harvests, and shall be sterile.

It is advisable to sterilize the bulk purified toxoid by filtration. A preservative such as thiomersal may be added to the bulk toxoid.
A.3.4.2 Sterility test

Each bulk purified toxoid shall be tested for bacterial sterility, according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (6, page 48).

A.3.4.3 Specific toxicity test

Each bulk purified toxoid shall be tested for the presence of diphtheria toxin by injection into at least 5 guinea-pigs, each weighing between 250 and 350 g. Each guinea-pig shall receive a subcutaneous injection with 1 ml of a dilution of purified toxoid containing at least 500Lf of toxoid. Animals that die shall be examined by autopsy for symptoms of diphtheria intoxication (red adrenals). The bulk purified toxoid shall pass the test if no guinea-pig shows symptoms of specific intoxication within 6 weeks of injection and if at least 80% of the animals survive the test period. The guinea-pigs shall not have been used previously for experimental purposes.

Some manufacturers carry out in addition a test for detecting the presence of diphtheria toxin by injecting intradermally into rabbits or guinea-pigs at least 20 Lf of purified toxoid and observing the animals for specific erythema.

A.3.4.4 Test for antigenic purity

Each bulk purified toxoid shall be tested for antigenic purity by determining the Lf value and the concentration of protein (nondialysable) nitrogen. The Lf determination shall be made by means of a reference material calibrated against the International Reference Preparation of Diphtheria Antitoxin for Flocculation Test or an equivalent reference preparation approved by the national control authority. The method of testing shall be approved by the national control authority. The bulk purified toxoid shall pass the test if it contains no fewer than 1500 Lf per mg of protein (nondialysable) nitrogen.

A.3.5 Control of final bulk

A.3.5.1 Preparation

The final bulk material shall be prepared from bulk purified toxoid. The number of Lf in a single human dose shall be approved
by the national control authority. Products intended for immunization of children shall contain between 10 and 50 LF in a single human dose. Plain diphtheria toxoid must not be used for primary immunization for children.

Products intended only for the immunization of adults may contain fewer than 10 LF in a single human dose. In some countries, such vaccines contain 2-5 LF per dose.

When intended for primary immunization the plain toxoid should be combined with pertussis vaccine, or a mineral adjuvant (aluminium or calcium carrier), or both.

A.3.5.2 Preservative

A suitable preservative shall be added to the final bulk. The preservative used shall have been shown in the amount present in the final bulk to have no deleterious effect on the toxoid or on other vaccine components with which the toxoid may be combined, and to cause no untoward reactions in man. The preservative and its concentration shall be approved by the national control authority.

A.3.5.3 Adjuvants

The adjuvant used, its purity, and its concentration shall be approved by the national control authority.

Aluminium or calcium compounds should be used as mineral carriers.

The concentration of aluminium shall not exceed 1.25 mg and that of calcium shall not exceed 1.3 mg per single human dose.

In some countries these upper limits for the concentrations of mineral carriers are considered to be too high and about half these concentrations are used.

In some countries the adsorbent is precipitated in the presence of the toxoid, in order to ensure better adsorption and higher stability.

A.3.5.4 Sterility test

Each final bulk shall be tested for bacterial sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (6, page 48).
A.3.5.5 Specific toxicity test

Each final bulk shall be tested for the presence of diphtheria toxin by injection into at least 5 guinea-pigs, each weighing between 250 and 350 g. Each guinea-pig shall receive a subcutaneous injection with a quantity equivalent to at least 5 single human doses. Animals that die shall be examined by autopsy for symptoms of diphtheria intoxication (red adrenals). The final bulk shall pass the test if no guinea-pig shows symptoms of specific intoxication within 6 weeks of injection and if at least 80% of the animals survive the test period.

A.3.5.6 Potency test

Each final bulk shall be tested for immunizing potency by comparison with a national reference material calibrated against the appropriate international standard. The test shall involve the inoculation of groups of guinea-pigs (weighing 250–350 g) and dilutions of both the final bulk and reference material shall be used. After immunization, the animals shall be challenged either with a lethal challenge dose of toxin given by the subcutaneous route or with graded doses of toxin given by the intradermal route. Standard statistical methods shall be used to calculate the potency of the final bulk. The method adopted and its interpretation shall be approved by the national control authority.

In some countries, potency testing is not carried out on each final bulk but on each final lot. In such cases, the provisions of Part A, section A.5.3, are applicable.

Sufficient animals should be used to achieve a 95% confidence interval smaller than 50–200%.

The details of suitable methods of potency testing are given in the Manual (4, Appendix D.11). More information, and hence greater precision, is obtained using the same number of animals with the multiple intradermal challenge method.

In some countries, potency is tested by mixing sera of immunized guinea-pigs with graded doses of toxin, followed by titration of the resulting mixtures in cell cultures.

The potency of the final bulk shall be approved by the national control authority. The potency of diphtheria vaccines containing an adjuvant and used for primary immunization of children shall be not less than 30 IU per single human dose. For this purpose, the reference
material used shall be one calibrated against the International Standard for Diphtheria Toxoid, Adsorbed.

Products intended only for booster immunization of adults may contain less than 30 IU per single human dose.

Plain diphtheria toxoid shall not be recommended for primary immunization.

A.3.5.7 Test for residual free detoxifying agents

Each final bulk shall be tested for residual free formaldehyde, if this has been used for detoxification, by a method approved by the national control authority, and it shall contain not more than 0.2 g/l of residual free formaldehyde.

A suitable test is a colorimetric determination of the reaction product of formaldehyde and fuchsin-sulfurous acid.

Similar tests for the presence of other detoxifying agents (glutaraldehyde) shall be included. The tests used and the limits of concentration of such chemicals shall be approved by the national control authority.

A.3.5.8 Stability test

The stability of the vaccine shall be shown to the satisfaction of the national control authority; at least 3 consecutive batches of final bulk shall be tested to prove stability during storage. When any changes in the production procedure are made, the vaccine produced by the new method shall be shown to be stable.

The vaccine shall meet the requirements for potency (see Part A, section A.3.5.6) up to the expiry date, provided that it has been stored at the recommended temperature.

Manufacturers should be encouraged to establish the time/temperature relationships of the stability with respect to potency.

A.3.5.9 Test for pH

The pH of the final bulk shall be recorded.

The pH should be between 6.0 and 6.7.
A.4 FILLING AND CONTAINERS

The requirements concerning filling and containers given in Part A, section 4, of Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 11) shall apply.

Single-dose and multipie-dose containers may be used. Attention is drawn to the risk associated with the use of the latter, and also to the fact that it is inadvisable for the interval between withdrawal of the first and final doses to be unduly prolonged. Therefore, the filling of an excessive number of doses into multiple-dose containers should be avoided.

It is recommended that, once a vial has been opened, it should be used up on the same day. If storage and reuse are absolutely unavoidable, the vial must be kept in a refrigerator at 5°C ± 3°C and never for more than one working week.

A.5 CONTROL TESTS ON FINAL PRODUCT

A.5.1 Identity test

An identity test shall be performed on at least one labelled container from each final lot.

Flocculation of the toxoid with diphtheria antitoxin may serve as an identity test. Tests on toxoid containing an aluminium or calcium carrier may be performed after the carrier has been dissolved in a solution of sodium citrate or after the toxoid has been eluted by a suitable method. If the carrier cannot be removed, tests may be made by specific antitoxin neutralization or by antitoxin production in animals.

A.5.2 Sterility test

Each final lot shall be tested for sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances) (6, page 48).

A.5.3 Potency test

A potency test shall be made according to Part A, section A.3.5.6 (page 49) on each final lot if such a test has not been performed on the final bulk.
A.5.4 Innocuity test

Each final lot shall be tested for abnormal toxicity by the injection of one human dose but not more than 1 ml into each of 5 mice (weighing 17–22 g) and at least one human dose but not more than 5 ml into each of 2 guinea-pigs (weighing 250–350 g) by the intraperitoneal route. The tests shall be approved by the national control authority. The final product shall be considered as innocuous if the animals survive for at least 7 days without showing significant signs of toxicity.

A.5.5 Test for adjuvant content

Each final lot shall be tested for adjuvant content by a method approved by the national control authority (see Part A, section A.3.5.3) if this test has not been performed on the final bulk.

In some countries, this test is applied to the final bulk only.

A.5.6 Test for preservative content

Each final lot shall be tested for the content of preservative (see Part A, section A.3.5.2). The test method shall be approved by the national control authority.

In some countries this test is applied to the final bulk only.

A.5.7 Test for pH

The pH of each final lot shall be recorded, if this test has not been done on the final bulk.

The pH should be between 6.0 and 6.7. In some countries this test is applied to the final bulk only.

A.5.8 Inspection of final containers

Each container in each final lot shall be inspected visually, and those showing abnormalities—such as clumping or the presence of particles—shall be discarded.

A.6 RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General
DIPHTHERIA

Requirements for Manufacturing Establishments and Control Laboratories (3, page 17) shall apply.

Written records shall be kept of all tests, irrespective of their result. The records shall be of a type approved by the national control authority.

A sample of a suitable summary protocol to be used for diphtheria toxoid is given on pages 55–60.

A.7 SAMPLES

The requirements given in Part, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 18) shall apply.

A.8 LABELLING

The label printed on or affixed to each container and the label on the carton enclosing one or more containers shall show at least:

— the words “Vaccinum Diphtheriae” and/or the proper name of the product;
— the word “adsorbed”, if applicable;
— the name and address of the manufacturer;
— the number of the final lot;
— the temperature of storage and the expiry date if kept at that temperature; and
— the recommended single human dose and route of administration.

In addition, the label printed on or affixed to the container or the label on the carton or the leaflet accompanying the container shall contain the following supplementary information:

— the fact that the toxoid fulfils the requirements of this document;
— the nature and amount of preservative present in the toxoid;
— the nature and amount of adsorbing agent (if applicable);
— the conditions recommended during storage and transport;
— an instruction that the adsorbed vaccine should not be frozen;
— an instruction that the adsorbed vaccine should be shaken before use; and
— instructions for the use of the toxoid and information about contraindications and the reactions that may follow vaccination.

A.9 DISTRIBUTION AND TRANSPORT

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 18) shall apply.

A.10 STORAGE AND EXPIRY DATE

The statements concerning storage temperature and expiry dates appearing on the label, as required in Part A, section 8, shall be based on experimental evidence and shall be submitted for approval to the national control authority.

A.10.1 Storage conditions

The manufacturer shall recommend such conditions of storage and transport as will ensure that the toxoid conforms to the requirements for potency until the expiry date stated on the label.

Storage at a temperature of 5°C ± 3°C has been found to be satisfactory.

A.10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory potency test, the date of the potency test being that on which the test animals were first inoculated with the toxoid. When toxoid is issued by the manufacturer, the expiry date shall not be more than 3 years from the beginning of the potency test.

PART B. NATIONAL CONTROL REQUIREMENTS

B.1 GENERAL

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1
(General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 19) shall apply.

The national control authority shall:
— specify the potency requirements;\(^3\)
— approve the method of manufacture;
— approve the method of detoxification and purification;
— approve the concentration of preservative and adjuvant;
— approve the test for stability; and
— provide the national reference materials.

### B.2 RELEASE AND CERTIFICATION

A toxoid shall be released only if it fulfils Part A of the present requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify that the lot of toxoid in question meets all national requirements as well as Part A of the present requirements. The certificate shall state the date of the last satisfactory potency test, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of diphtheria toxoid between countries.

**SUMMARY PROTOCOL FOR DIPHTHERIA TOXOID PRODUCTION AND TESTING**

**Identification of Final Lot**

Name and address of manufacturer

\(^3\) The specified laboratory potency test referred to in section A.3.5.6 (page 49) should be such that, when the product is used for primary immunization in man according to the recommended schedule (see footnote on page 41), the geometric mean antibody response not less than 6 months after completion of primary immunization should be at least 0.25 IU of antitoxin per millilitre of serum.
Diphtheria

Lot No.

Date of manufacture of final lot

Nature of final product (plain or adsorbed)

Volume of recommended single human dose

No. of containers in final lot for each filling volume

Information on Manufacture

1. Strain

Identity of *C. diphtheriae* strain used in vaccine

2. Single harvests included in final bulk *

   - Medium
     
     Period of incubation
     
     Date of earliest harvest included
     
     Conditions of storage

3. Bulk purified toxoid

   Results of test for antigenic purity
   (Lf/mg protein N)

4. Final bulk

   Date of preparation

   Lf per ml

   Results of test for residual free formaldehyde

   pH

5. Adjuvant

   Nature

   mg/Al or Ca

---

* A list of the identification numbers of the single harvests and bulk purified toxoids should be included.
6. Preservative
   Nature
   Concentration in final product
   (by assay or calculation)

7. Buffer
   Concentration

Tests on Final Bulk

1. Sterility
   Date of test and results
   Was a repeat test necessary?

2. Specific toxicity
   No. of animals
   Date of injection
   Dose of toxoid injected (Lf per animal)
   Route of injection
   Period of test
   Results of test
   Bulk purified toxoid
   Final bulk or final lot

3. Potency
   Weight of animals
   No. of animals per dose of toxoid
   Date of immunization and volume of dilutions administered
   Date of challenge or bleed
   Challenge dose
   Date of end of test
DIPHTHERIA

Results of lethal challenge test (if done)

<table>
<thead>
<tr>
<th>Dilation</th>
<th>No. of survivors</th>
<th>ED50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference toxoid (IU/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test toxoid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Potency of test toxoid: IU per single human dose
95% confidence limits of potency

Results of multiple intradermal test (if done)

<table>
<thead>
<tr>
<th>Dilation</th>
<th>Mean score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference toxoid (IU/ml)</td>
<td></td>
</tr>
<tr>
<td>Test toxoid</td>
<td></td>
</tr>
</tbody>
</table>

Potency of test toxoid: IU per single human dose
95% confidence limits of potency
1. **Identity**

   Test for diphtheria toxoid and results
   Test for pertussis vaccine and results
   Test for tetanus toxoid and results

2. **Sterility**

   No. of containers examined
   Method of test
   Date of start of test
   Date of end of test
   Results

3. **Potency**

   If this test has not been performed on the final bulk, report these data in the space provided under "Tests on final bulk".

4. **Inocuity**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Guinea-pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td></td>
</tr>
<tr>
<td>Route of injection</td>
<td></td>
</tr>
<tr>
<td>Volume of injection</td>
<td></td>
</tr>
<tr>
<td>Date of start of test</td>
<td></td>
</tr>
<tr>
<td>Date of end of test</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td></td>
</tr>
</tbody>
</table>

5. **Preservative**

   Concentration of preservative

6. **pH**

   Results of pH test
   Signature of head of laboratory
Certification by person taking overall responsibility for production of the vaccine:
I certify that lot No. of the vaccine satisfies Part A of the WHO Requirements for Diphtheria Toxoid.

Signature

Name typed

The protocol must be accompanied by a sample of the label and a copy of the leaflet.

REQUIREMENTS FOR PERTUSSIS VACCINE

GENERAL CONSIDERATIONS

The formulation of the Requirements for Pertussis Vaccine and the events leading up to those requirements have already been described (1, Annex 1). The vaccines have been in use on a wide scale for almost 20 years and, where vaccines of known potency have been used correctly in schedules of known efficacy, the incidence of whooping cough has decreased markedly.

Recently, however, two factors have raised doubts in the minds of public health administrators. These concern the toxicity of the vaccine and an apparent lack of efficacy. Pertussis vaccine is a suspension of killed organisms and the inoculation of up to \(20 \times 10^9\) whole organisms has given rise to toxic reactions. There appears to be no immediate prospect that a nontoxic and effective vaccine can be produced against whooping cough and, until the protective antigen against pertussis has been isolated and characterized, this situation is unlikely to change. In the meantime, it is possible to produce vaccines with good potency and that provoke few adverse reactions. The aim of these requirements is to encourage the production of such vaccines as well as to indicate where further research may be helpful. Clearly, the ultimate goal is to produce a nontoxic, highly potent vaccine.

Parapertussis also gives rise to paroxysmal cough and may be confused with true pertussis. In a few countries, therefore, a parapertussis component is added to the vaccine.

It is mandatory for all pertussis vaccines to be shown to be potent by the mouse protection test. The best evidence that this test
correlates with clinical efficacy came from the Medical Research Council trial carried out in the United Kingdom from 1948 to 1954, from which it was concluded that vaccines shown to protect mice against intracerebral challenge also protected immunized children against whooping cough when such children were exposed to the disease in the home by infection from a sibling. The establishment of this correlation was a marked advance in the development of whole bacterial vaccines against whooping cough, but there were a number of anomalies that still demand an explanation. For this reason failure to protect mice against intracerebral challenge should not preclude the clinical evaluation of new preparations. Until an alternative test has been shown to correlate with efficacy in man, however, the mouse protection test will continue to be recognized as the only test for the measurement of potency. The test is included in these requirements.

Much effort has been made in attempts to obtain more reproducible results in the mouse protection test. The use of clean mice selected at random for their place in the test has improved the uniformity of results. The selection of the particular strain of mice has also affected the results and great attention should be paid to these details of the test.

Although the mouse protection test for potency continues to be the only recognized measure of efficacy, research should continue and be actively encouraged towards the establishment of alternative or supplementary tests.

The immunizing dose of pertussis vaccine is an arbitrary guess at the number of killed organisms that are required to give an adequate antigenic stimulus in order to induce protection. The number of organisms in the vaccine required for such a purpose is measured by the opacity of the bacterial suspension. Over the last decade the International Reference Preparation of Opacity has been a suspension of minute particles of glass suspended in water. The fifth International Reference Preparation of Opacity, however, is a plastic rod in which one plastic is suspended in another in the fluid state before the rod becomes solid. Such rods simulate the appearance of a bacterial suspension and it is important that the fifth International Reference Preparation of Opacity should be adopted by all countries and that the opacity of bacterial suspensions should be expressed in International Units of Opacity.

Tests for toxicity continue to pose a problem. Many toxins may be produced during the growth of pertussis organisms. Some of these,
such as the heat-labile necrotizing toxin, may be measured quite accurately, whereas others are difficult to quantify. The present requirements can do no more than adopt the mouse weight-gain test that has been used as an "in process" control measure adopted by many manufacturers and required by some control authorities. This should not be an impediment to further developments in the measurement of the toxicity of pertussis vaccines.

It is known that some strains and the medium in which they are grown may give rise to toxic vaccines and that slight changes in the growth conditions may reduce toxicity. It is not known which toxin gives rise to reactions in children, and the identification of such a toxin is needed urgently.

Attempts are being made to produce extract vaccines in order to obtain a vaccine free from local and systemic reactions. Although the characteristics of such vaccines may be measured in the laboratory, restrictions in the testing of new vaccines have made it almost impossible to measure their efficacy in young children. It is necessary to bear in mind that such studies are essential, and assistance should be given to the producers of extract vaccines to have them tried in areas in which whooping cough is endemic. The present formulation of international requirements is based on the methods currently in use for the preparation and control of killed whole bacterial vaccines.

An improvement that can be made in the stability of the vaccines is to use bacterial strains that have been shown to yield vaccines more stable in the presence of heat than those produced normally. Suggestions have been made to include suitable tests for the selection of strains for vaccine production. It is hoped that such selection will give rise to vaccines with increased heat stability.

Investigations are continuing on the production of freeze-dried, adsorbed DPT vaccine. Encouraging results have been obtained with the trial lots and a consistency record is now being established in some countries.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in type of normal size have been written in the form of requirements so that, if a health administration so desires, these parts as they appear may be used as definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning pertussis vaccine, it
is recommended that a clause be included permitting modifications of the manufacturing requirements on condition that such modified requirements ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements formulated below. The World Health Organization should then be informed of the action taken.

The terms "national control authority" and "national control laboratory", as used in these requirements, always refer to the country in which the vaccine is manufactured.

PART A. MANUFACTURING REQUIREMENTS

A. 1. DEFINITION

A.1.1. International name and proper name

The international name shall be "Vaccinum Pertussis". The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

A.1.2. Descriptive definition

Vaccinum Pertussis is a saline suspension of killed Bordetella pertussis. The preparation shall satisfy all the requirements formulated below.

A.1.3. International standard, international reference preparation, and international units

The International Standard for Pertussis Vaccine, established in 1957 (7, page 5), is dispensed in ampoules containing 52 mg of dried vaccine. The International Unit (4, page 11) is defined as the activity contained in 1.5 mg of the International Standard.

Each ampoule therefore contains 34.7 IU.

The fifth International Reference Preparation of Opacity, established in 1975 (9, page 16), consists of a plastic rod simulating the optical properties of a bacterial suspension (10 IU of opacity).

The International Standard for Pertussis Vaccine is in the custody of the International Laboratory for Biological Standards, State Serum Institute, Copenhagen. Samples are distributed, free of charge, on request to national control laboratories. The International Standard is intended for the
calibration of national standards and reference preparations for use in the manufacture and laboratory control of pertussis vaccine.

The International Reference Preparation of Opacity is in the custody of the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London.

A.1.4 Terminology

Single harvest: A suspension of bacteria prepared from the cultures of one strain of B. pertussis inoculated, harvested, and processed together.

Final bulk: The homogeneous finished vaccine present in a single container from which the final containers are filled either directly or indirectly through one or more intermediate containers.

Final lot: A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling. A final lot must, therefore, have been filled from a single container in one working session.

A.2 GENERAL MANUFACTURING REQUIREMENTS

The general requirements for manufacturing establishments contained in Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, Annex 1) shall apply to establishments manufacturing pertussis vaccine, with the addition of the following.

Written descriptions of procedures for the preparation of pertussis vaccine adopted by a manufacturer shall be submitted for approval to the national control authority. Proposals for modifications shall be submitted for approval to the national control authority before their implementation.

A.3 PRODUCTION CONTROL

A.3.1 Control of source materials

A.3.1.1 Strains of B. pertussis

Strains of B. pertussis used in preparing vaccine shall be identified by a full record of their history, including their origin,
characters on isolation, and particulars of all tests made periodically for verification of strain characters. The strains shall be chosen in such a way that the final vaccine includes agglutinogens 1, 2, and 3.

Recent work has shown that some strains of *B. pertussis* give more stable vaccine than others. Furthermore, some strains are more toxic than others. Trial vaccines should be made from single strains, therefore, using the medium of growth and method of inactivation to be used in the production of vaccines. Heat stability and toxicity tests should be applied to these single strain vaccines. Ideally the strains giving the least toxic and most stable and potent vaccines should be selected for vaccine production.

The strains shall be maintained by a method that will preserve their ability to yield potent vaccine.

Freeze-drying or storage in liquid nitrogen is a satisfactory method of maintaining strains.

A.3.1.2 Seed lot system

The production of pertussis vaccine shall be based on a seed lot system. Cultures of the working seed shall have the same characteristics as the strain from which the parent seed lot was derived.

A.3.1.3 Culture medium for production of bacteria

The medium (*10, Appendix P.1*) shall be capable of growing *B. pertussis*, retaining agglutinogens and potency. The medium shall also be one that does not give rise to a toxic vaccine.

It is recognized that *B. pertussis* vaccine is toxic, but some media give rise to more toxic vaccine than others.

The use of human blood in the medium is contraindicated because of the possibility of introducing the agent of serum hepatitis into the vaccine. If blood from animals is used, precautions should be taken to remove blood constituents by a suitable method.

If a fluid medium that will form part of the finished vaccine is used, it is particularly important that it should be free from ingredients known to cause toxic or allergic reactions in man.

A.3.2 Production precautions

The general production precautions, as formulated in the requirements of Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufac-
turing Establishments and Control Laboratories) (3, page 15) shall apply to the manufacture of pertussis vaccine.

Suitable methods for the production of pertussis vaccine are given in the Manual for the Production and Control of Vaccines: Pertussis Vaccine (10, section P. 7).

A.3.3 Control of single harvests

Production shall be shown to be consistent by observing the growth rate, pH, and agglutinogen content of the culture.

Any culture showing anomalous growth characteristics should be investigated and shown to be satisfactory before being accepted as a single harvest.

A.3.3.1 Control of bacterial purity

Samples of single harvests taken before killing shall be tested for purity by microscopic examination of stained smears and by inoculation into appropriate culture media. Single harvests shall not be used for the final bulk unless they have been shown to be free from contaminating organisms and to have the morphological appearance typical of pertussis bacteria.

A.3.3.2 Opacity

The opacity of each single harvest shall be determined not later than 2 weeks after harvesting and before the bacterial suspension has been subjected to a process capable of altering its opacity. The opacity shall be determined in comparison with the International Reference Preparation of Opacity or an equivalent reference preparation approved by the national control authority.

A suspension of pertussis organisms that on visual inspection has an opacity approximately equal to 10 IU of opacity may be considered for practical purposes to have a concentration of 10,000 million organisms per ml.4

4 The concentration determined by direct count, however, would probably be less than 10,000 million per ml. The question of relationship of international units of opacity to concentration of organisms has been considered by the Expert Committee on Biological Standardization (WHO Technical Report Series, No. 96, 1955, p. 8, and No. 259, 1963, p. 26).
A.3.3.3 Killing and detoxification

After sampling for purity control and opacity testing, the bacteria shall be killed and detoxified by a method approved by the national control authority. If chemicals are used for this treatment, they shall be approved by the national control authority. In order to ensure that the organisms are killed, a sample shall be tested using an appropriate culture medium.

Heating at 56°C for 30 min is an effective method of killing the bacteria and destroying much of the heat-labile toxins. Chemicals such as formaldehyde and thiomersal also are used successfully for this treatment. Their effectiveness depends on the concentration as well as on the temperature, time, and pH at which the treatment is carried out. Since this is an important stage in the manufacture of vaccine, manufacturers should satisfy themselves, on the basis of tests with their own particular product, that the method used is optimal in that it achieves detoxification while retaining adequate potency and stability.

A.3.4 Control of the final bulk

A.3.4.1 Preparation

The final bulk shall be prepared by pooling a number of single harvests. Where vaccine is made from two or more strains, the consecutive batches of the final bulk shall be consistent with respect to the proportions of each strain present as measured by opacity. The material shall be diluted with buffered physiological saline (pH 7.0) or other suitable diluent, so that the opacity of the final bulk in the volume recommended as a single human dose does not exceed 20 IU of opacity if the human dose is 1 ml or 40 IU of opacity if the human dose is 0.5 ml. The number of opacity units in the final bulk shall be calculated from the number of opacity units determined in the test (described in Part A, section A.3.3.2) performed on the single harvests.

It is advisable to keep the opacity as low as possible while still fulfilling the potency requirements outlined in section A.3.4.6

In some countries, vaccines containing an "aluminium carrier" are not permitted to exceed 16 IU of opacity if the human dose is 1 ml or 32 IU of opacity if the human dose is 0.5 ml.
A.3.4.2 Preservative

A suitable preservative shall be added to the final bulk. The preservative shall have been shown to have no deleterious effect on the vaccine or on other vaccine components with which the vaccine may be combined, and to cause no untoward reactions in man. The preservative and its concentration shall be approved by the national control authority.

A.3.4.3 Adjuvants

Adjuvants may be added to the vaccine in concentrations approved by the national control authority. The chemicals used shall be approved by the national control authority.

Aluminium or calcium compounds should be used as mineral carriers.

The concentration of aluminium shall not exceed 1.25 mg and that of calcium shall not exceed 1.3 mg per single human dose.

In some countries these upper limits for the concentration of mineral carriers are considered to be too high, and about half these concentrations are used.

A.3.4.4 Sterility test

Each final bulk shall be tested for bacterial sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances, Revised 1973) (6, page 48).

A.3.4.5 Toxicity test

Each final bulk shall be tested for toxicity by a method approved by the national control authority.

There is little information on the relationship between toxicity of vaccines in animals and the occurrence of untoward reactions following vaccination in man. Toxicity tests in animals are required by many national control authorities, but in most tests the results obtained depend to some extent on factors independent of the vaccine, such as the strain of animal used and the conditions under which the animals are kept. One of the tests most widely used is the mouse toxicity test. This has been of some value in ensuring the production of vaccines which, in
general, are satisfactory in that they cause minimal untoward reactions in man. This test may be performed as follows.

No fewer than 10 healthy mice are used, each weighing 14–16 g. They should have access to food and water for at least 2 hours before injection and continuously after injection for the duration of the test. The total weight of the group of mice is determined immediately before injection. The mice used for testing the vaccine(s) and the control group of mice should be of the same sex. If both sexes are used, they should be equally distributed in all groups. Each mouse is given an intraperitoneal injection with 0.5 ml containing a volume of the final bulk under test that is equivalent to not less than half the largest volume recommended as a single human dose. A similar control group of mice is inoculated with 0.5 ml of physiological saline, preferably containing the same concentration of the same preservative as that present in the final bulk vaccine. The total weight of each group of mice is determined at the end of 72 h and at the end of 7 days after injection. The final bulk is considered satisfactory if (a) at the end of 72 h the total weight of the group is not less than that preceding the injection, (b) at the end of 7 days the average weight gain per mouse is not less than 60% of that in the control group of mice, and (c) not more than 5% of the mice die.

Since the toxicity of pertussis vaccine is not well understood, manufacturers and national control laboratories should continue to study tests for toxicity in order to understand it better.

A.3.4.6 Potency test

The potency of each final bulk (or of each final lot) shall be determined by a comparative assay in relation to that of a reference material calibrated against the International Standard for Pertussis Vaccine or an equivalent standard approved by the national control authority. The assay shall be performed by the intracerebral mouse protection test and the method shall be approved by the national control authority.

The potency of the final bulk shall be not less than 4 IU in the volume recommended as a single human dose.

A satisfactory method of carrying out the assay is as follows:

(a) Mice. Healthy mice, preferably from a strain and colony capable of giving an adequate immune response, are used. Preferably, they should be of the same sex but, if this is not possible, both sexes should be distributed equally throughout the test and the sexes are segregated. The weight of each mouse should be at least 10 g and not more than 15 g, and in a single test the individual weights of the mice should not vary by more than 4 g. A system of randomization of the mice is employed with regard to their distribution into groups, shelf position of cages,
order of immunization, and order of challenge. For this purpose
a table of random numbers or other suitable means may be
used.

At least 48 mice are used for each vaccine under test and at
least 48 for the standard vaccine. They are distributed into three
groups each of at least 16 mice for the vaccine under test, and
into three groups each of at least 16 mice for the standard
vaccine. At least 40 mice are used for control purposes. These are
distributed into four groups each of at least 10 mice: one group
for the challenge dose and three for titrating the challenge
suspension in order to determine the ratio of the challenge dose
to the LD₅₀ — i.e., the dose of challenge suspension estimated to
kill 50% of the mice.

(b) Immunization of mice. Three fivefold serial dilutions in
physiological saline are made of the final bulk and also of the
standard vaccine. The dilutions are chosen in each case so as to
include the immunizing dose expected to be 30% effective (LD₃₀
or IM LD₃₀). Each mouse in each group for immunization is given
an intraperitoneal injection with 0.5 ml of the appropriate
dilution.

c) The challenge. The interval between immunization and
challenge is 14-17 days. At least 94% of the mice should survive
and remain healthy during this interval.

The strain used for challenge (generally W18-323) should be
approved by the national control authority. To ensure constancy
of virulence from test to test, a large lot of master culture is
dispensed into ampoules and freeze-dried or stored in liquid
nitrogen. Each challenge suspension is prepared from a single
freeze-dried culture taken from the master lot.

The bacterial suspension for challenge is prepared from a
20-24-hour culture grown on Bordet-Gengou medium or other
suitable medium that has been seeded from a rapidly growing
culture not more than 30 h old. Alternatively, aliquots of the
challenge suspension may be frozen and kept in liquid nitrogen.
Such aliquots are thawed, diluted, and used directly as the
challenge culture. The suspension is diluted with a diluent
capable of maintaining the viability of the organisms. A satis-
factory diluent is an aqueous solution containing 10 g/l casein
peptone and 6 g/l sodium chloride adjusted to a pH of 7.1 ± 0.1.
The suspension, free from particles of agar or clumps of bacteria,
is adjusted in such a way that the challenge dose of 0.05 ml
contains 100-500 LD₅₀. It has been shown that the variation
between a challenge dose ranging from 100 to 1000 LD₅₀ does
not affect the vaccine potency found. This may be done conve-
niently by first adjusting the suspension to an opacity equivalent
to that of the International Reference Preparation and then
diluting it accordingly.

The immunized mice are challenged under a mild ether
anesthesia by intracerebral injection of the challenge dose. Lastly,
the challenge dose and its dilutions are injected into the control
mice by the intracerebral route. Syringes fitted with needles of nominal external diameter 0.40 mm (27 gauge) are used for the intracerebral injections.

The interval between the preparation of the challenge suspension and injection into the last mouse does not exceed 2.5 h. As a check on the viability of the challenge suspension, a colony count is made after completing the injections, by cultivating the dilutions used for the control mice. The colony count is usually about one-quarter as great as would be expected on the basis of the opacity determination (see section A.3.3.2).

(d) Record of results. The mice are observed for 14 days. Mice dying within 3 days are excluded from the test.

(e) The ED₉₀ values for each preparation are determined by a method of statistical analysis that must include the transformation of the mouse survival data into a form capable of consistently producing a linear regression. Probits, logits, and angle transformation have been shown to be suitable. Similar methods of analysis should be used to determine the LD₉₀ of the challenge suspension.

(f) Validity of the test. The test is valid if the ED₉₀ of each vaccine is between the largest and the smallest immunizing doses, the regressions are linear, and the limits of one standard error of each ED₉₀ fall within a range of 64–156% of the immune response is graded in relation to the immunizing doses, and the regressions do not show significant deviations from parallelism and linearity (P = 0.05).

(g) Estimate of potency. The ED₉₀ of the vaccine under test and the standard vaccine are calculated by a method that provides an estimate of the standard error or of the 95% fiducial limits. The potency is estimated in terms of International Units in the volume that is recommended for a single human dose. The vaccine passes the requirements for potency if the result of a statistically valid test shows that the estimate of potency of the vaccine is equal to or more than 4 IU in the volume recommended for a single human dose. If the potency is below this value, the vaccine fails the potency requirements. One additional test may be made, and in this case the vaccine passes the requirement for potency only if the geometric mean of the best estimates of both potency values found in statistically valid tests is equal to or more than 4 IU in the volume recommended for a single human dose.

A.3.4.7 Stability test

The stability of the vaccine shall be shown to the satisfaction of the national control authority: at least three consecutive batches of

³ This range has been calculated on the assumption that 16 mice per group are included and that the slope of the log-dose/response line, calculated by probit analysis, is 1.0.
final bulk shall be tested to prove stability during storage. When any changes in the production procedure are made, the vaccine produced by the new method shall be shown to be stable.

The vaccine shall meet the requirements for potency (see Part A, section A.3.4.6) up to the expiry date, provided that it has been stored at the recommended temperature.

Manufacturers should be encouraged to establish the time/temperature relationships of the stability with respect to potency.

A.3.4.8 Test for pH

The pH of the final bulk shall be recorded.

The pH should be 7.0 ± 0.3.

A.4 FILLING AND CONTAINERS

The requirements concerning filling and containers given in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 16) shall apply.

Single-dose and multiple-dose containers may be used. Attention is drawn to the risk associated with the use of the latter, and also to the fact that it is inadvisable for the interval between withdrawal of the first and final doses to be unduly prolonged.

The filling of an excessive number of doses into multiple-dose containers should therefore be avoided.

It is recommended that, once a vial has been opened, it should be used up on the same day. If storage and reuse are absolutely unavoidable, the vial must be kept in a refrigerator at 5°C ± 3°C, and never for longer than one working week.

A.5 CONTROL TESTS ON FINAL PRODUCT

A.5.1 Identity test

An identity test shall be performed on at least one container from each final lot.

Agglutination of the organisms with specific antipurussis serum may serve as an identity test. Vaccines may also be inoculated into animals in order to show the presence of pertussis agglutinins in their serum.
A.5.2 Sterility test

Each final lot shall be tested for sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances) (6, page 48).

A.5.3 Potency test

A potency test shall be made according to Part A, section A.3.4.6, on each final lot if such a test has not been made on the final bulk.

A.5.4 Innocuity test

Each final lot shall be tested for abnormal toxicity by injecting, by the intraperitoneal route, a human dose into each of 5 mice and 5 human doses into each of 2 guinea-pigs. The final product shall be considered as innocuous if the animals survive for at least 7 days without showing significant signs of toxicity. The tests shall be approved by the national control authority.

A.5.5 Test for adjuvant content

Each final lot shall be tested for adjuvant content by a method approved by the national control authority (see Part A, section A.3.4.3) if this test has not been performed on the final bulk.

In some countries, this test is applied to the final bulk only.

A.5.6 Test for preservative content

Each final lot shall be tested for the content of preservative (see Part A, section A.3.4.3). The test method shall be approved by the national control authority.

In some countries, this test is applied to the final bulk only.

A.5.7 Test for pH

The pH of each final lot shall be recorded.

The pH should be 7.0±0.3. In some countries, this test is applied to the final bulk only.
A.5.8 Inspection of final containers

Each container in each final lot shall be inspected visually, and those showing abnormalities — such as clumping or the presence of particles — shall be discarded.

A.6 RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 17) shall apply, with the addition of the following.

Written records shall be kept of all tests irrespective of their results. The records shall be of a type approved by the national control authority.

A sample of a suitable summary protocol to be used for pertussis vaccine is given on pages 77-80.

A.7 SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 18) shall apply.

A.8 LABELLING

The label printed on or affixed to each container, and the label on the carton enclosing one or more containers, shall show at least:

— the words “Vaccinum Pertussis” and/or the proper name of the product;
— the word “adsorbed”, if applicable;
— the name and address of the manufacturer;
— the temperature of storage and the expiry date if kept at that temperature; and
— the volume of the recommended human dose.

In addition, the label printed on or affixed to the container, or the label on the carton, or the leaflet accompanying the container, shall contain the following additional information:

— the fact that the vaccine fulfils the requirements of this document;
— the nature and amount of preservative present in the vaccine;
— the nature and amount of adsorbing agent (if applicable);
— the nature and amount of any additional substances present in the vaccine;
— the conditions recommended during storage and transport;
— an instruction that the vaccine should not be frozen;
— an instruction that the vaccine should be shaken before use;
— the recommended single human dose and route of administration; and
— instructions for the use of the vaccine and information about contraindications and the reactions that may follow vaccination.

A.9 DISTRIBUTION AND TRANSPORT

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (A, page 18) shall apply.

A.10 STORAGE AND EXPIRY DATE

The statements concerning storage temperature and expiry date appearing on the label, as required in Part A, section A.8, shall be based on experimental evidence and shall be submitted for approval to the national control authority.

A.10.1 Storage conditions

The manufacturer shall recommend such conditions of storage and transport, and shall ensure that the vaccine conforms to the requirements of potency until the expiry date stated on the label.

Storage at a temperature of 5°C ± 3°C has been found to be satisfactory. The vaccine should not be frozen.

A.10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory potency test, the date of the potency test being that on which the test animals were inoculated with the vaccine. When vaccine is issued by the manufacturer, the expiry date shall not be more than 2 years from the beginning of the potency test.
PART B. NATIONAL CONTROL REQUIREMENTS

B.1 GENERAL

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 19) shall apply.

In view of the lack of information on the relationship between toxicity of vaccines in animals and the production of untoward reactions in man after vaccination, and the wide fiducial limits of the test for potency (Part A, sections A.3.4.5 and A.3.4.6), the degree of consistency in producing satisfactory final bulk vaccines is an important factor in ensuring the safety and efficacy of a particular manufacturer's product. Definite requirements in this respect cannot be formulated, but it is advisable that the national control authorities satisfy themselves, from the results of tests on a series of consecutive final bulk vaccines, that the manufacturer is able to reach a satisfactory consistency of quality of the product.

The national control authority shall:
— approve the method of manufacture;
— approve the method of inactivation;
— approve the nature and concentration of adjuvant and preservative;
— approve the test for toxicity;
— approve the details of the method of potency assay;
— approve the test for stability; and
— provide the national reference materials.

B.2 RELEASE AND CERTIFICATION

A vaccine shall be released only if it fulfils Part A of the present requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify that the lot of vaccine in question meets all national requirements as well as Part A of the present requirements. The certificate shall state the date of the last satisfactory potency test, the lot number, the number under which the lot was released, and the number appearing on the labels of the
containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of pertussis vaccine between countries.

SUMMARY PROTOCOL FOR PERTUSSIS VACCINE PRODUCTION AND TESTING

Identification of Final Lot

Name and address of manufacturer

Lot No.

Date of manufacture of final lot

Nature of final product (plain or adsorbed)

Volume of recommended single human dose

No. of containers in final lot for each filling volume

Information on Manufacture

1. Strain

Identity of B. pertussis strain used in vaccine

Serological type of strain

Date(s) of reconstitution of ampoule(s) for manufacture

2. Single harvests included in final bulk *

Medium

Date of inoculation

Date of harvest

Method of killing

* A list of the identification numbers of the single harvests and bulk suspensions should be included.
3. Bulk material
Method of killing
Results of tests for living organisms
Date of preparation of final bulk
Volume of final bulk

4. Adjuvant
Nature
mg/AI or Ca
Volume added

5. Preservative
Nature
Concentration in final product
(by assay or calculation)

6. Buffer
Concentration

Tests on Final Bulk

1. Sterility
Date of test and results
Was a repeat test necessary?

2. Toxicity
Species, weight, and sex of animals
No. of animals
Date of injection
Route of injection
Period of test
Results of test
3. Potency

- Weight and sex of mice
- No. of mice per dose of vaccine
- Date of immunization
- Challenge dose
- Date of challenge
- Date of end of test

Results of potency test

<table>
<thead>
<tr>
<th>Dilution</th>
<th>No. of survivors</th>
<th>ED$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference vaccine (IU/ml)</td>
<td></td>
<td>ml</td>
</tr>
<tr>
<td>Test vaccine</td>
<td></td>
<td>ml</td>
</tr>
</tbody>
</table>

Potency of test vaccine ______ IU per single human dose
95% confidence limits of potency

Tests on Final Lot

1. Identity

- Test for diphtheria toxoid and results
- Test for pertussis vaccine and results
- Test for tetanus toxoid and results

2. Sterility

- No. of containers examined
- Method of test
3. **Potency**

If this test has not been performed on the final bulk, report these data in the space provided under "Tests on final bulk".

4. **Inocuity**

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Guinea-pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Route of injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume of injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date of start of test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date of end of test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5. **Preservative**

Concentration of preservative

6. **pH**

Results of pH test

Signature of head of laboratory

Certification by person taking overall responsibility for production of the vaccine:
I certify that lot No. ... of the vaccine satisfies Part A of the WHO Requirements for Pertussis Vaccine.

Signature

Name typed

The protocol must be accompanied by a sample of the label and a copy of the leaflet.
TETANUS

REQUIREMENTS FOR TETANUS TOXOID

GENERAL CONSIDERATIONS

Tetanus toxoid is one of the most immunogenic antigens available for protection against an infectious disease. In the developed countries its use has markedly decreased the demands for tetanus antitoxin, but in the developing world much needs to be done to increase its availability. This is especially the case where tetanus neonatorum can be eliminated by the immunization of pregnant women.

The developments leading to the formulation of the first Requirements for Tetanus Toxoid are described in detail in the Introduction and General Considerations to the Requirements for Biological Substances No. 10, (2, Annex 1). The purpose of these General Considerations is to draw attention to the significant developments that have taken place since those Requirements were formulated.

As with diphtheria toxoid, the most important consideration is the agreement reached on the formulation of requirements for the assay of potency. It has now been accepted that the potency of tetanus toxoid can be measured by an active challenge test and that either guinea-pigs or mice may be used. A comparison has been made of the use of a lethal challenge dose and a paralytic challenge dose and both give similar results when the potency of a test vaccine is compared with that of a reference preparation. It is interesting to note, however, that when pertussis vaccine is mixed with tetanus toxoid the adjuvant effect of pertussis is more marked in mice than in guinea-pigs. This difference must be recognized when assaying a combined vaccine for the potency of the tetanus component. However, the minimum acceptable level of potency expressed in International Units must be specified by the national control authority.

Since the last Requirements were formulated in 1964, the International Standard for Tetanus Antitoxin has been replaced. Furthermore, it has been shown that this second International Standard has an in vivo : in vitro ratio of 1.4:1.0 and may be used for the flocculation test, assuming that each ampoule contains 1000Lf equivalents. In addition, an International Standard for Tetanus Toxoid, Adsorbed, was established in 1965. This was required because the dose-response relationship for adsorbed products was
not parallel to that for the plain (nonadsorbed) products. This standard has been most useful in the establishment of national standards required for the quality control of adsorbed products of which tetanus toxoid is a component. It is important that countries should adopt the principle of expressing the potency of tetanus toxoid in International Units rather than in Lf, which may give misleading information.

These requirements have introduced a test for stability because of the need to ensure that vaccines that may have been subjected to high ambient temperatures will retain their potency.

Although there are few data to permit the correlation of a potency level in a biological assay with protection in man, and even fewer for the correlation between the potency level and the duration of immunity, sufficient evidence is available to assign a minimum potency level above which a vaccine may be considered to be of acceptable potency. Such levels have been incorporated into the present Requirements.

In the present International Requirements for Tetanus Toxoid, purification of the product is required. Tetanus toxoid in the unpurified form is liable to give vaccination reactions in man, and much work has been done in developing purified material to avoid such reactions. However, even with purified products, untoward reactions may occur in adults. There is evidence that purification, although enabling more concentrated preparations to be used, may sometimes reduce the immunizing activity of tetanus toxoid, probably on account of the removal of substances having an adjuvant effect. Such purified products may be used for primary immunization, but it is preferable to combine them either with pertussis vaccine containing whole organisms or with a mineral adjuvant (or both).

Each of the following sections constitutes a recommendation. The parts of each section that are printed in type of normal size have been written in the form of requirements, so that, if a health administration so desires, these parts as they appear may be used as definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance.

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6 Primary immunization refers to the initial course of injections, which usually consists of two (or three) injections at an interval of 4–6 weeks, followed by a further injection 7–12 months later. Reinforcing immunity, which is sometimes referred to as "boosting" immunity, refers to subsequent single injections, usually given a number of years later.
Should individual countries wish to adopt these requirements as the basis of their national regulations concerning tetanus vaccine, it is recommended that a clause be included permitting modifications of the manufacturing requirements on condition that such modified requirements ensure that the degree of safety and the potency of the toxoids are at least equal to those provided by the requirements formulated below. The World Health Organization should then be informed of the action taken.

The terms "national control authority" and "national control laboratory", as used in these requirements, always refer to the country in which the toxoid is manufactured.

PART A. MANUFACTURING REQUIREMENTS

A.1 DEFINITION

A.1.1 International name and proper name

The international name shall be "Vaccinum Tetani". The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to toxoids that satisfy the requirements formulated below.

A.1.2 Descriptive definition

Vaccinum Tetani is a preparation of tetanus toxoid prepared by treating tetanus toxin with formaldehyde. The preparation shall satisfy all the requirements formulated below.

A.1.3 International Standards and International Units

The second International Standard for Tetanus Antitoxin (established in 1969) is stored in ampoules containing dried hyperimmune horse serum containing 1400 IU per ampoule.

This standard has an in vivo/in vitro ratio of 1.4:1.0 and may be used for the flocculation test, assuming that each ampoule contains 1000 IU equivalents.

The expression of the International Unit as a given weight of a dried substance contained in an ampoule is for the purposes of definition only. In practice the International Reference materials should be used by reconstituting the whole contents of the ampoules in a given volume and using the reconstituted material with a calculated number of units per millilitre depending on the volume of fluid used and the declared content of each ampoule.
The International Standard for Tetanus Toxoid, Plain (established in 1951), is dispensed in dried form in ampoules containing 833 IU. The International Unit is defined as the activity contained in 0.03 mg of the International Standard.

The International Standard for Tetanus Toxoid, Adsorbed (established in 1965), is dispensed in ampoules containing 80 mg of tetanus toxoid adsorbed to aluminium hydroxide, plus an equal part of guinea-pig serum, dried (120 IU per ampoule). The International Unit is defined as the activity contained in 0.6667 mg of the International Standard.

The above-mentioned standards are in the custody of the International Laboratory for Biological Standards, State Serum Institute, Copenhagen. Samples are distributed free of charge on request to national control laboratories. The international standards are intended for the calibration of national standards for use in the manufacture and laboratory control of tetanus antitoxin and toxoid.

A.1.4 Terminology

Single harvest. The toxic filtrate or toxoid obtained from one batch of cultures inoculated, harvested, and processed together.

Bulk purified toxoid. The processed purified material, prepared from either a single harvest or a pool of a number of single harvests. It is the parent material from which the final bulk is prepared.

Final bulk. The final homogeneous toxoid present in a single container from which the final containers are filled either directly or through one or more intermediate containers.

Final lot. A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling. Therefore, a final lot must have been filled from a single container in one working session.

A.2 GENERAL MANUFACTURING REQUIREMENTS

The general requirements for manufacturing establishments contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, Annex I) shall apply to establishments manufacturing tetanus toxoid, with the addition of the following:

All manufacturing processes up to and including the completion of detoxification shall take place in completely separate areas by means of separate equipment.
Written descriptions of procedures for the preparation of tetanus toxoid adopted by a manufacturer shall be submitted for approval to the national control authority. Proposals for modifications shall be submitted for approval to the national control authority before their implementation.

A.3 PRODUCTION CONTROL

A.3.1 Control of source materials

A.3.1.1 Strains of Clostridium tetani

Strains of *C. tetani* used in preparing tetanus toxoid shall be identified by a record of their history and of all tests made periodically for verification of strain characters.

A highly toxigenic strain of *C. tetani* should be used. A strain that has proved satisfactory in many laboratories is the Harvard strain (No. 49205). Special attention should be paid to maintaining the strain either by freeze-drying or by selective subculturing in order to ensure retention of its toxigenic properties.

A.3.1.2 Seed lot system

The production of tetanus toxin shall be based on a seed lot system. Cultures of the working seed shall have the same characteristics as the cultures of the strain from which the parent seed lot was derived. The preparation of the seed lot shall be in compliance with the requirements Part A, section A.3.2.

A.3.1.3 Culture medium for production of toxin

The medium shall be free from ingredients that will be present in the final product and that are known to cause toxic or allergic reactions in man.

Since some medium components may be present in the finished product, it is particularly important to ensure that the final product is free from substances that are likely to cause toxic or allergic reactions in man. If the medium is prepared from protein digest, as for example casein hydrolysate or digested muscle, precautions should be taken to ensure that the digestion
has proceeded sufficiently to free the medium from such substances. Neither mammalian protein nor human blood group substances should be present in the final vaccine; the method for detecting these substances should be approved by the national control authority.

A.3.2 Production precautions

The general production precautions, as formulated in the requirements of Part A, Section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 15) shall apply to the manufacture of tetanus toxoid.

Suitable methods for the production of tetanus toxoid are given in the Manual for the production and control of vaccines: tetanus toxoid (11, Section T.7).

A.3.3 Control of single harvests

The production shall be shown to be consistent by observing the growth, pH, and rate of toxin production.

Any culture showing anomalous growth characteristics should be investigated and shown to be satisfactory before being accepted as a single harvest.

A.3.3.1 Control of bacterial purity

Samples of the cultures used for preparing a single harvest shall be tested for bacterial purity by microscopic examination of stained smears and by inoculation into appropriate culture media. Single harvests shall not be used for preparing bulk material if contamination has occurred at any stage in their preparation.

A.3.3.2 Filtration

After sampling for control of purity, the culture shall be filtered by means of a filter capable of producing a bacteriologically sterile filtrate.

The cultures may be treated with a killing agent (formaldehyde) before filtration. On no account should phenol be used.

If they are not so treated, the cultures should be filtered as soon as possible after the end of their incubation period. To facilitate filtration, the cultures may be centrifuged and/or a filter aid added beforehand.

In some countries any filter shedding fibres may not be used.
A.3.3.3 Determination of the concentration of toxin (Lf)

The supernatant of the whole culture prior to inactivation shall be tested by flocculation or other suitable test approved by the national control authority.

Flocculation is performed in comparison with a reference material calibrated against the second International Standard for Tetanus Antitoxin or an equivalent reference preparation approved by the national control authority.

The time taken for flocculation to occur should be recorded. It has been shown that toxins or toxoids that take an abnormally long time to flocculate are frequently poor in immunizing potency.

It is advisable also to determine the Lf dose together with the minimal lethal dose for mice.

Suitable methods for these determinations are described in the Manual (II, Appendices T.6 and T.7).

It is preferable that toxic filtrates used in preparing purified toxoid should contain 40 Lf/ml or more.

These tests are not absolute measures of potency, but they are a good guide to the consistency of production.

A.3.3.4 Detoxification and purification of toxin

The purification process may precede or follow detoxification and the method shall be approved by the national control authority. The agent used shall fulfill the requirements of the International Pharmacopoeia (5, page 228) or other specifications approved by the national control authority.

Any free formaldehyde shall be removed after the completion of detoxification. The method used shall be approved by the national control authority.

Care should be taken to ensure that reversal to toxin does not take place on storage. A method for the detection of reversal is suggested in the Manual (II, Appendices T.6 and T.7).

In some countries the following test is applied to check the possibility of toxicity reversal. One sample is diluted to 200 Lf/ml (with 0.0167 mol/l phosphate-buffered saline) and another sample to a concentration equivalent to that of the final bulk. The latter sample is kept standing at 37°C for 20 days. Then 5 ml of each sample are injected subcutaneously into each of at least 5 guinea-pigs, which are then observed for 21 days (as described in Part A, Section A.3.4.3).
The method of purification shall be such that no substances likely to cause untoward reactions in man are incorporated into the final product.

A.3.4 Control of bulk purified toxoid

A.3.4.1 Preparation

The bulk purified toxoid shall be prepared from either a single harvest or a pool of a number of single harvests, and shall be sterile.

It is advisable to sterilize the bulk purified toxoid by filtration. A preservative such as thioglycol may be added to the bulk toxoid.

A.3.4.2 Sterility test

Each bulk toxoid shall be tested for bacterial sterility according to the requirements given in Part A, Section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (6, page 48).

A.3.4.3 Specific toxicity test

Each bulk purified toxoid shall be tested for the presence of tetanus toxin by injection into at least 5 guinea-pigs, each weighing 250–350 g. Each guinea-pig shall be given a subcutaneous injection with 1 ml of a dilution of toxoid containing at least 500 Lf of toxoid. Animals that die shall be examined by autopsy. The bulk toxoid shall pass the test if no guinea-pig shows symptoms of specific paralysis or any other signs of tetanus within 21 days of injection and if at least 80% of the animals survive the test period. The guinea-pigs shall not have been used previously for experimental purposes.

A.3.4.4 Test for antigenic purity

Each bulk purified toxoid shall be tested for antigenic purity by determining the Lf value and the concentration of protein (non-dialysable) nitrogen. The Lf determination shall be made with a reference material calibrated against the second International Standard for Tetanus Antitoxin or an equivalent reference preparation approved by the national control authority. The method
of testing shall be approved by the national control authority. The bulk purified toxoid shall pass the test if it contains no fewer than 1000 Lf per mg of protein (nondialysable) nitrogen.

Measurement of the total combining power (TCP) is also an indication of the quality of the antigen when expressed in relation to the Lf content. A suitable method for measuring the TCP is given in the Manual (11, Appendix T.10).

A.3.5 Control of final bulk

A.3.5.1 Preparation

The final bulk material shall be prepared from bulk purified toxoid. The amount of toxoid contained in a single human dose shall be approved by the national control authority and be such that the requirement for potency is fulfilled (see Part A, Section A.3.5.5).

A.3.5.2 Preservative

A suitable preservative shall be added to the final bulk. The preservative shall have been shown in the amount present in the final bulk to have no deleterious effect on the toxoid or on other vaccine components with which the toxoid may be combined, and to cause no untoward reactions in man. The preservative and its concentration shall be approved by the national control authority.

A.3.5.3 Adjuvants

The adjuvant used, its purity, and its concentration shall be approved by the national control authority.

Aluminium or calcium compounds should be used as mineral carriers.

The concentration of aluminium shall not exceed 1.25 mg and that of calcium shall not exceed 1.3 mg per single human dose.

In some countries these upper limits for the concentration of mineral carriers are considered to be too high and about half these concentrations are used.

In some countries the adsorbent is precipitated in the presence of the toxoid in order to obtain better adsorption and higher stability.
A.3.5.4 Sterility test

Each final bulk shall be tested for bacterial sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances, Revised 1973) (6, page 48).

A.3.5.5 Specific toxicity test

Each final bulk shall be tested for the presence of tetanus toxin by injection into at least 5 guinea-pigs, each weighing 250-350 g. Each guinea-pig shall be given a subcutaneous injection with a quantity equivalent to at least 5 single human doses. Animals that die shall be examined by autopsy. The final bulk shall pass the test if no guinea-pig shows paralysis or any other signs of tetanus within 21 days of injection and if at least 80% of the animals survive the test period.

A.3.5.6 Potency test

Each final bulk shall be tested for immunizing potency by comparison with a national reference material calibrated against the appropriate international standard. The test shall involve the inoculation of groups of guinea-pigs (weighing 250-350 g) or mice (weighing 14–20 g, provided that, in a single test, the individual weights of the mice shall not vary by more than 3 g). Three dilutions of both the final bulk and reference material shall be used. After immunization, the animals shall be challenged with a lethal or paralytic challenge dose of toxin given by the subcutaneous route. Standard statistical methods shall be used to calculate the potency of the final bulk. The method adopted and its interpretation shall be approved by the national control authority.

In some countries potency testing is not carried out on each final bulk but on each final lot. In such cases, the provisions of Part A, Section A.5.3, page 48, are applicable.

Sufficient animals should be used to achieve a 95% confidence interval smaller than 50–200%.

The details of suitable methods for potency testing are given in the Manual (11, Appendix T.13).

The potency of the final bulk shall be approved by the national control authority. The potency of tetanus vaccine containing an adjuvant shall be not less than 40 IU per single human dose. For this
purpose the reference material used shall be one calibrated against the International Standard for Tetanus Toxoid, Adsorbed.

Plain tetanus toxoid may be used for primary immunization but there is no agreed potency expressed in International Units for this preparation. Therefore, the potency should be approved by the national control authority.

A.3.5.7 Test for residual free formaldehyde

Each final bulk shall be tested for residual free formaldehyde, if this has been used for detoxification, by a method approved by the national control authority, and shall contain not more than 0.2 g/l of residual free formaldehyde.

A suitable test is a colorimetric determination of the reaction product of formaldehyde and fuchsia-sulfurous acid.

A.3.5.8 Stability test

The stability of the vaccine shall be shown to the satisfaction of the national control authority: at least three consecutive batches of final bulk shall be tested to prove stability during storage. When any changes in the production procedure are made, the vaccine produced by the new method shall be shown to be stable.

The vaccine shall meet the requirements for potency (see Part A, Section A.3.5.6) up to the expiry date, provided that it has been stored at the recommended temperature.

Manufacturers should be encouraged to establish the time/temperature relationships of the stability with respect to potency.

A.3.5.9 Test for pH

The pH of the final bulk shall be recorded.

The pH should be between 6.0 and 6.7.

A.4 FILLING AND CONTAINERS

The requirements concerning filling and containers given in Part A, Section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 16) shall apply.
Single-dose and multiple-dose containers may be used. Attention is drawn to the risk associated with the use of the latter, and also to the fact that it is inadvisable for the interval between withdrawal of the first and final doses to be unduly prolonged. The filling of an excessive number of doses into multiple-dose containers should therefore be avoided.

It is recommended that, once a vial has been opened, it should be used up on the same day. If storage and reuse are absolutely unavoidable, the vial must be kept in a refrigerator at 5°C±3°C and never for more than one working week.

A.5 CONTROL TESTS ON FINAL PRODUCT

A.5.1 Identity test

An identity test shall be performed on at least one labelled container from each final lot.

Flocculation of the toxoid with tetanus antitoxin may serve as an identity test. Tests on toxoid containing an aluminium or calcium carrier may be performed after the carrier has been dissolved with a solution of sodium citrate or after the toxoid has been eluted by a suitable method. If the carrier cannot be removed, tests may be made by specific antitoxin neutralization or by antitoxin production in animals.

A.5.2 Sterility test

Each final lot shall be tested for sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances) (6, page 48).

A.5.3 Potency test

A potency test shall be made according to Part A, Section A.3.5.6 (p. 90), on each final lot if such a test has not been performed on the final bulk.

A.5.4 Innocuity test

Each final lot shall be tested for abnormal toxicity by the injection of one human dose but not more than 1 ml into each of 5 mice (weighing 17–22 g) and at least one human dose but not more than 5 ml into each of 2 guinea-pigs (weighing 250–350 g), by the
intraperitoneal route. The tests shall be approved by the national control authority. The final product shall be considered as innocuous if the animals survive for at least 7 days without showing significant signs of toxicity.

A.5.5 Test for adjuvant content

Each final lot shall be tested for adjuvant content by a method approved by the national control authority (see Part A, Section A.3.5.3) if this test has not been performed on the final bulk.

In some countries, this test is applied to the final bulk only.

A.5.6 Test for preservative content

Each final lot shall be tested for the content of preservative (see Part A, section A.3.5.2). The test method shall be approved by the national control authority.

In some countries, this test is applied to the final bulk only.

A.5.7 Test for pH

The pH of each final lot shall be recorded.

The pH should be between 6.0 and 6.7. In some countries, this test is applied to the final bulk only.

A.5.8 Inspection of final containers

Each container in each final lot shall be inspected visually, and those showing abnormalities—such as clumping or the presence of particles—shall be discarded.

A.6 RECORDS

The requirements given in Part A, Section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 17) shall apply.

Written records shall be kept of all tests irrespective of their results. The records shall be of a type approved by the national control authority.

A sample of a suitable summary protocol to be used for tetanus toxoid is given on pages 96-100.
A. 7 SAMPLES

The requirements given in part A, Section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 18) shall apply.

A. 8 LABELLING

The label printed on or affixed to each container, and the label on the carton enclosing one or more containers, shall show at least:
— the words "Vaccinum Tetani" and/or the proper name of the product;
— the word "adsorbed", if applicable;
— the name and address of the manufacturer;
— the number of the final lot;
— the temperature of storage and the expiry date if kept at that temperature; and
— the recommended single human dose and route of administration.

In addition, the label printed on or affixed to the container, or the label on the cartons, or the leaflet accompanying the container, shall contain the following information:
— the fact that the toxoid fulfils the requirements of this document;
— the nature and amount of preservative present in the toxoid;
— the nature and amount of adsorbing agent (if applicable);
— the conditions recommended during storage and transport;
— an instruction that the adsorbed vaccine should not be frozen;
— instructions for the use of the toxoid and information about contraindications and the reactions that may follow vaccination;
— an instruction that the adsorbed vaccine should be shaken before use.

A. 9 DISTRIBUTION AND TRANSPORT

The requirements given in Part A, Section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (3, page 18) shall apply.
A.10 STORAGE AND EXPIRY DATE

The statements concerning storage temperature and expiry date appearing on the label, as required in Part A, Section A.8, shall be based on experimental evidence and shall be submitted for approval to the national control authority.

A.10.1 Storage conditions

The manufacturer shall recommend such conditions of storage and transport as will ensure that the toxoid conforms to the requirements of potency until the expiry date stated on the label. Adsorbed vaccines shall not be frozen.

Storage at a temperature of $5^\circ \pm 3^\circ C$ has been found to be satisfactory.

A.10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory potency test, the date of the potency test being that on which the test animals were first inoculated with the toxoid. When toxoid is issued by the manufacturer, the expiry date shall not be more than 3 years from the beginning of the potency test.

PART B. NATIONAL CONTROL REQUIREMENTS

B.1 GENERAL

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirement for Manufacturing Establishments and Control Laboratories) (3, page 19) shall apply.

The national control authority shall:

- specify the potency requirements;\(^5\)
- approve the method of manufacture;

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\(^5\) The specified laboratory potency test referred to in section A.3.5.6 (page 90) should be such that, when the product is used for primary immunization in man according to the recommended schedule (see footnote on page 82), the geometric mean antibody response not less than 6 months after completion of primary immunization should be at least 0.5 IU of antitoxin per millilitre of serum.
— approve the method of detoxification and purification;
— approve the concentration of preservative and adjuvant;
— approve the test for stability; and
— provide the national reference materials.

B.2 RELEASE AND CERTIFICATION

A toxoid shall be released only if it fulfills Part A of the present requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify that the lot of toxoid in question meets all national requirements as well as Part A of the present requirements. The certificate shall state the date of the last satisfactory potency test, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of tetanus toxoid between countries.

SUMMARY PROTOCOL FOR TETANUS TOXOID
PRODUCTION AND TESTING

Identification of Final Lot

Name and address of manufacturer

Lot No.

Date of manufacture of final lot

Nature of final product (plain or adsorbed)

Volume of recommended single human dose

No. of containers in final lot for each filling volume

Information on Manufacture

1. Strain

Identity of Clostridium tetani strain used in vaccine
2. Single harvests included in final bulk *
   
   Medium
   Period of incubation
   Date of earliest harvest included
   Conditions of storage

3. Bulk toxoid
   Purified/Unpurified
   Nature of bulk toxoid
   Results of test for antigenic purity, if applicable (Lf/mg protein N)

4. Final bulk
   Date of preparation
   LF per ml
   Results of test for residual free formaldehyde
   pH

5. Adjuvant
   Nature
   mg/Al or Ca

6. Preservative
   Nature
   Concentration in final product (by assay or calculation)

7. Buffer
   Concentration

---

* A list of the identification numbers of the single harvests and bulk purified toxoids should be included.
TETANUS
Tests on Final Bulk

1. Sterility
   Date of test and results
   Was a repeat test necessary?

2. Specific toxicity
   No. of animals
   Date of injection
   Dose of toxoid injected (Lf per animal)
   Route of injection
   Period of observation
   Results of test

3. Potency
   Species of animals
   Weight and sex of animals
   No. of animals per dose of toxoid
   Date of immunization and volume of dilutions administered
   Date of challenge or bleed
   Challenge dose
   Date of end of test

Results of challenge test

<table>
<thead>
<tr>
<th>Dilution</th>
<th>No. of survivors</th>
<th>ED_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference toxoid (1U/ml)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Tests on Final Lot

1. Identity
   Test for diphtheria toxoid and results
   Test for pertussis vaccine and results
   Test for tetanus toxoid and results

2. Sterility
   No. of containers examined
   Method of test
   Date of start of test
   Date of end of test
   Results

3. Potency
   If this test has not been performed on the final bulk, report these data in the space provided under "Tests on final bulk".

4. Immunity
   Mice
   Guinea-pigs
   No. of animals
   Route of injection
   Volume of injection
   Date of start of test
   Date of end of test
   Results
5. **Preservative**

Concentration of preservative

6. **pH**

Results of pH test

Signature of head of laboratory

Certification by person taking overall responsibility for production of the vaccine:

I certify that lot No. ____ of the vaccine satisfies Part A of the WHO Requirements for Tetanus Toxoid.

Signature

Name typed

The protocol must be accompanied by a sample of the label and a copy of the leaflet.

**GENERAL CONSIDERATIONS FOR COMBINED VACCINES**

There are no specific requirements for combined vaccines. In order to satisfy the need for such requirements, national control authorities have combined the appropriate tests for the single components, but there has been no uniformity between the various authorities.

The requirements for a combined vaccine must take into consideration the tests applicable to the bulk materials that are incorporated into the final product. Many tests of the single components are applied at the bulk stage and, after blending, further tests are required, as is the case with the final filled material. The need for tests at each stage in the production is important because of the interaction between antigens, as well as the effects that both adjuvants and preservatives may have on the potency and stability of the final product.

The tests applied to the combined vaccines are the same as those applied to the single components. Therefore, cross-reference to
the tests specified for the individual components is appropriate. Some tests are common for both combined vaccines diphtheria–pertussis–tetanus (DPT) and diphtheria–tetanus (DT), but other tests — such as that for potency assay — need special consideration, particularly when mice are used for the assay.

There are no international reference preparations specifically for the combined vaccines, and the potency of each component is expressed in international units by comparison with reference materials calibrated against the reference materials for the single components. This is not an ideal situation, because the dose–response relationships in animals differ when the components are in the combined form. Nevertheless, meaningful potency data are obtained, and attention has not been given to the establishment of reference materials for combined vaccines because there has not been a demand for them so far.

The expiry date of combined vaccines is determined by the component with the shortest expiry period.

A number of manufacturers and control authorities have experienced difficulties in reporting the results of tests of combined vaccines. Therefore a composite protocol for this purpose has been included for DPT.

These requirements have been restricted to the two combined vaccines DPT and DT, since these are the most widely used.

No attempt has been made to include other combinations, such as cholera or typhoid vaccine mixed with tetanus toxoid. Requirements for these vaccines, therefore, should include the relevant tests for the individual components, and special attention should be given to possible interactions that may occur between the components.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in type of normal size have been written in the form of requirements so that, if a health administration so desires, these parts as they appear may be used as definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning combined vaccines, it is recommended that a clause be included permitting modifications of manufacturing requirements on condition that such modified requirements ensure that the degree of safety and the potency of the vaccines are at least equal to those provided by the requirements
formulated below. The World Health Organization should then be
informed of the action taken.

The terms “national control authority” and “national control
laboratory”, as used in these requirements, always refer to the
country in which the vaccine is manufactured.

PART A. MANUFACTURING REQUIREMENTS

TESTS FOR ALL COMBINED VACCINES

The following requirements are common to diphtheria, pertussis,
and tetanus vaccines (DPT) and to diphtheria and tetanus vaccines
(DT), as applied to the final bulk.

A.1 Test for adjuvant content

The requirements for diphtheria (section A.3.5.3, page 48),
which are the same as those for pertussis (section A.3.4.3, page 68)
and for tetanus (section A.3.5.3, page 89), shall apply to all
combined vaccines to which an adjuvant has been added.

A.2 Sterility test

The requirements for diphtheria (section A.3.5.4, page 48),
which are the same as for pertussis (section A.3.4.4, page 68) and
for tetanus (section A.3.5.4, page 90), shall apply to all combined
vaccines.

A.3 Free detoxifying agent

The requirements for diphtheria (section A.3.5.7, page 50) shall
apply to all combined vaccines.

A.4 Stability test

The requirements for diphtheria (section A.3.5.8, page 50) which
are the same as for pertussis (section A.3.4.7, page 71) and for
tetanus (section A.3.5.8, page 91), shall apply to all combined
vaccines.

The following requirements are common to both DPT and DT as
applied to the final lot.
A.5 Sterility test

The requirements for diphtheria (section A.5.2, page 51), which are the same as for pertussis (section A.5.2, page 73) and for tetanus (section A.5.2, page 92), shall apply to all combined vaccines.

A.6 Test for adjuvant content

The requirements for diphtheria (section A.5.5, page 52), which are the same as for pertussis (section A.5.5, page 73) and for tetanus (section A.5.5, page 93), shall apply to all combined vaccines.

A.7 Test for preservative content

The requirements for diphtheria (section A.5.6, page 52), which are the same as for pertussis (section A.5.6, page 73) and for tetanus (section A.5.6, page 93), shall apply to all combined vaccines.

A.8 Inspection of final containers

The requirements for diphtheria (section A.5.8, page 52), which are the same as for pertussis (section A.5.8, page 74) and for tetanus (section A.5.8, page 93), shall apply to all combined vaccines.

SPECIAL TESTS FOR DPT VACCINE

The following tests shall apply to DPT in the final bulk.

A.9 Potency test

For the tests for the potency of the diphtheria component, the requirements for diphtheria (section A.3.5.6, page 49) shall apply.

For the test for the potency of the pertussis component, the requirements for pertussis (section A.3.4.6, page 69) shall apply.

For the test for the potency of the tetanus component, the requirements for tetanus (section A.3.5.6, page 90) shall apply, with the following addition.

The potency of the tetanus component shall not be less than 40 IU per single human dose if the test is performed in guinea-pigs. If
mice are used, the potency of the tetanus component shall be 60 IU per single human dose. The 95% confidence interval of the tests shall be smaller than 50–200%.

A.10 Specific toxicity test

For the test for diphtheria specific toxicity, the requirements for diphtheria (section A.3.5.5, page 49) shall apply.

For the test for tetanus specific toxicity, the requirements for tetanus (section A.3.5.5, page 90) shall apply.

For these two tests the same animals are used and are observed for 6 weeks in order to cover the observation period specified for diphtheria (section A.3.5.5, page 49).

For the test for pertussis toxicity, the requirements for pertussis (section A.3.4.5, page 68) shall apply.

The following tests shall apply to DPT in the final lot.

A.11 Identity test

The identity test of diphtheria and tetanus toxoid shall be made in a triple vaccine after the centrifugation of the pertussis organisms. The tests specified in the requirements for diphtheria (section A.5.1, page 51) and for tetanus (section A.5.1, page 92) shall apply.

The tests for the toxoids in a triple vaccine containing an adjuvant are made after dissolving the mineral carrier with sodium citrate and then centrifuging the pertussis organisms.

For the tests for the identity of the pertussis component, the test specified in the requirements for pertussis (section A.5.1, page 72) shall apply.

A.12 Potency test

If a test for potency has not been performed on the triple vaccine in the final bulk, the tests as specified in A.9 shall apply.

A.13 Innocuity test

The test for innocuity shall be made according to the requirements for pertussis (section A.5.4, page 73).
A.14 Test for pH

The pH of DPT vaccine shall be 6.7–7.0.

SPECIAL TESTS FOR DT VACCINE

The following tests shall apply to DT vaccine in the final bulk.

A.15 Potency test

The test for potency of the diphtheria component is that specified for diphtheria (section A.3.5.6, page 49).

The test for potency of the tetanus component is that specified for tetanus (section A.3.5.6, page 90).

A.16 Specific toxicity test

The test for specific toxicity of diphtheria and for tetanus toxicity is as specified for combined vaccines (section A.10, page 104).

The following tests shall apply to DT vaccine in the final lot.

A.17 Identity test

The identity of diphtheria and tetanus toxoid shall be tested as specified for diphtheria (section A.5.1, page 51) and for tetanus (section A.5.1, page 92).

The tests for DT adsorbed vaccine shall be made after dissolving the mineral carrier with sodium citrate.

A.18 Potency test

If a test for potency has not been performed on the final bulk, the tests specified for combined vaccines (section A.15 above) shall apply.

A.19 Innocuity test

The test for innocuity shall be made according to the requirements for diphtheria (section A.5.4, page 52) or for tetanus (section A.5.4, page 92).
A.20 Test for pH

The pH of the DT vaccine shall be 6.0–7.0.

PART B. NATIONAL CONTROL REQUIREMENTS

In addition to the responsibilities included under each individual vaccine, the national control authority shall:
- approve the formulation of the combined vaccine to ensure that the components are present at concentrations appropriate to its use;
- approve the formulation with the preservative and adjuvant to ensure that the vaccine is stable up to the expiry date, provided that it has been stored at the recommended temperature; and
- approve the protocols for reporting the results of tests applied to the combined vaccine. A suggested protocol for this purpose is given below.

SUMMARY PROTOCOL FOR DIPHTHERIA–PERTUSSIS–
TETANUS VACCINE

Identification of Final Lot

Name and address of manufacturer

Lot No.

Date of manufacture of final lot

Nature of final product (plain or adsorbed)

Volume of recommended single human dose

No. of containers in final lot for each filling volume

DIPHTHERIA TOXOID

Information on Manufacture

1. Strain

Identity of *C. diphtheriae* strain used in vaccine
2. Single harvests included in final bulk

- Medium
- Period of incubation
- Date of earliest harvest included
- Conditions of storage

3. Bulk purified toxoid

- Results of test for antigenic purity (Lf/mg protein N)

PERTUSSIS VACCINE
Information on Manufacture

1. Strains

- Identity of B. pertussis strain used in vaccine
- Serological type of strain
- Date(s) of reconstitution of ampoule(s) for manufacture

2. Single harvests included in final bulk

- Medium
- Date of inoculation
- Date of harvest
- Method of killing
- Opacity
- Conditions of storage

3. Bulk material

- Results of tests for living organisms

* A list of the identification numbers of the single harvests and bulk purified toxoids should be included.
1. **Strain**
   Identity of *C. tetani* strain used in vaccine

2. **Single harvests included in final bulk**
   - **Medium**
   - **Period of incubation**
   - **Date of earliest harvest included**
   - **Conditions of storage**

3. **Bulk toxoid**
   Purified/Unpurified
   - **Nature of bulk toxoid**
   - **Results of test for antigenic purity, if applicable (Lf/mg protein N)**

**INFORMATION ON BLENDING**

Composition of the Final Bulk

1. **Identity of diphtheria toxoid component**
   - **Lf/ml**
   - **Volume added**

2. **Identity of tetanus toxoid component**
   - **Lf/ml**
   - **Volume added**

3. **Identity of pertussis vaccine component**
   - **Opacity units**
   - **Volume added**

*A list of the identification numbers of the single harvests and bulk purified toxoids should be included.*
4. Adjuvant
   - Nature and concentration
   - mg/Al or Ca in final bulk
   - Volume added

5. Preservative
   - Nature
   - Concentration in final product (by assay or calculation)

6. Buffer
   - Concentration

7. Date of completion of final bulk
   - Volume of final bulk

Tests on Final Bulk

1. Sterility
   - Date of test and results
   - Was a repeat test necessary?

2. Specific toxicity
   - Date of test for diphtheria toxoid and results
   - Date of test for pertussis vaccine and results
   - Date of test for tetanus toxoid and results

3. Potency
   (i) Diphtheria
   - Weight of animals
   - No. of animals per dose of toxoid
### COMBINED VACCINES

- **Date of immunization and volume of dilutions administered**
- **Date of challenge or bleed**
- **Challenge dose**
- **Date of end of test**

(a) **Results of lethal challenge test (if done)**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>No. of survivors</th>
<th>ED$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference toxoid (IU/ml)</td>
<td>ml</td>
<td></td>
</tr>
<tr>
<td>Test toxoid</td>
<td>ml</td>
<td></td>
</tr>
</tbody>
</table>

Potency of test toxoid __________ IU per single human dose

95% confidence limits of potency

(b) **Results of intradermal challenge test (if done)**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Mean score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference toxoid (IU/ml)</td>
<td></td>
</tr>
<tr>
<td>Test toxoid</td>
<td></td>
</tr>
</tbody>
</table>

Potency of test toxoid __________ IU per single human dose

95% confidence limits of potency
(ii) Pertussis

<table>
<thead>
<tr>
<th>Weight and sex of mice</th>
<th>No. of mice per dose of vaccine</th>
<th>Date of immunization</th>
<th>Challenge dose</th>
<th>Date of challenge</th>
<th>Date of end of test</th>
</tr>
</thead>
</table>

Results of challenge test

<table>
<thead>
<tr>
<th>Dilation</th>
<th>No. of survivors</th>
<th>$ED_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference vaccine (1 IU/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test vaccine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Potency of test vaccine: 1 U per single human dose

95% confidence limits of potency

(iii) Tetanus

<table>
<thead>
<tr>
<th>Species of animals</th>
<th>Weight of animals</th>
<th>No. of animals per dose of toxoid</th>
<th>Date of immunization and volume of dilutions administered</th>
<th>Date of challenge or bleed</th>
<th>Challenge dose</th>
<th>Date of end of test</th>
</tr>
</thead>
</table>
COMBINED VACCINES

Results of challenge test

<table>
<thead>
<tr>
<th>Dilution</th>
<th>No. of survivors</th>
<th>$ED_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference toxoid (1U/ml)</td>
<td></td>
<td>ml</td>
</tr>
<tr>
<td>Test toxoid</td>
<td></td>
<td>ml</td>
</tr>
</tbody>
</table>

Potency of test toxoid: [x] IU per single human dose

95% confidence limits of potency

TESTS ON FINAL LOT

1. Identity

   Test for diphtheria toxoid and results
   Test for pertussis vaccine and results
   Test for tetanus toxoid and results

2. Sterility

   No. of containers examined
   Method of test
   Date of start of test
   Date of end of test
   Results

3. Potency

   If this test has not been performed on the final bulk, report these data in the space provided under "Tests on final bulk".
4. **Isocuity**

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Guinea-pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Route of injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume of injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date of start of test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date of end of test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5. **Preservative**

Concentration of preservative

6. **pH**

Results of pH test

Signature of head of laboratory

Certification by person taking overall responsibility for production of the vaccine:

I certify that lot No. of the vaccine satisfies Part A of the WHO Requirements for Diphtheria Toxoid, Pertussis Vaccine, and Tetanus Toxoid.

Signature

Name typed

The protocol must be accompanied by a sample of the label and a copy of the leaflet.

**AUTHORS**

The Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines were prepared by the following WHO consultants and staff members:

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Dr P. Knight, Wellcome Research Laboratories, Beckenham, England (Consultant)
ACKNOWLEDGEMENTS

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REFERENCES

INTRODUCTION

The formulation of international requirements for the manufacture and control of BCG vaccine was first considered by the WHO Expert Committee on Biological Standardization in its thirteenth report (1, page 12). In its fourteenth report (2, page 12), the
Committee requested WHO to make arrangements as early as possible for the formulation of such requirements. These requirements were approved by the WHO Expert Committee on Biological Standardization at its eighteenth meeting and appeared as Annex 1 to its report (3).

In the intervening 12 years there have been a number of developments in the production and control of BCG vaccine. The majority of manufacturers are now producing only freeze-dried vaccine. Where the use of liquid BCG vaccine is permitted, it is on the responsibility of the national control authority. In this case the parts of these requirements that are appropriate should apply.

Much more is known concerning the production of freeze-dried vaccines from seed lots, which have been shown to yield satisfactory vaccines, and consideration must be given to the different routes and methods of administration. Furthermore the dose of living organisms causing tuberculin sensitivity without giving rise to untoward reactions has been carefully considered.

These revised requirements were drafted by a group of WHO consultants and staff members (listed on page 146). Account has been taken of the regulations and requirements for the manufacture and control of dried BCG vaccine in a number of countries, as well as information from published and unpublished reports. In addition, opinions and data have been received from a number of experts, whose assistance is acknowledged (see page 146).

GENERAL CONSIDERATIONS

BCG vaccine has been used for many years in a number of countries, and there is considerable evidence that the immunization of man with BCG vaccine by the intradermal or percutaneous routes can give a substantial degree of protection against tuberculosis, and that the inconveniences and risks associated with the vaccination are insignificant (seventh report of the WHO Expert Committee on Tuberculosis) (4, page 9). In the eighth report of the WHO Expert Committee on Tuberculosis (5) the concept of a comprehensive tuberculosis control programme on a countrywide scale was proposed, and in its ninth report (6) the Committee noted that, in spite of difficulties, some countries had been successful in implementing national tuberculosis programmes.
The formulation of the original international requirements for dried BCG vaccine was complicated by the following: (a) a number of different daughter strains derived from the original strain of the bacillus of Calmette and Guérin are used; (b) a number of different manufacturing and testing procedures are employed; (c) there is no proved laboratory method of assaying the protective potency of vaccines against tuberculous infection in man; (d) vaccines of different total bacterial content and number of culturable particles are produced; and (e) vaccines intended for administration by different routes are prepared. Although much is known about the characteristics of a good vaccine, several of these difficulties remain.

SCOPE OF THESE REQUIREMENTS

The revised requirements that have now been formulated refer to dried BCG vaccine prepared from strains derived from the bacillus of Calmette and Guérin. The requirements have been formulated primarily to cover vaccine intended for intradermal and percutaneous administration. Immunization with BCG vaccine by the oral route is questionable and this practice has been stopped in almost all countries. These requirements, therefore, are not intended to cover BCG vaccines given by this route.

Although the latest findings from controlled field trials have been taken into consideration in formulating these revised requirements, there are still a number of characteristics needing further study. In particular, the maximum dose of viable organisms in the various vaccines that can be tolerated without producing an unacceptably high incidence of untoward reactions must be determined by careful studies in the various age groups. Guidance may be given by WHO on these matters, but the studies must be carried out in the communities in which the vaccines are to be used.

BCG STRAINS

Various strains, all derived from the bacillus of Calmette and Guérin, are at present being used by manufacturing establishments in the preparation of BCG vaccine. While these strains differ from one another to minor degrees when investigated in the laboratory, there is
no evidence to suggest that they differ significantly in their power to protect man. There is no strain that is known to be definitely superior to the others. Only seed lots that have been shown to be acceptable by laboratory and clinical tests should be used. Several such seed lots are available through WHO. It is believed that a suitable seed lot of BCG should yield vaccines that give protection in experimental animals and produce long-lasting tuberculin sensitivity in human beings, but with only a low frequency of untoward reactions.

Most of the early trials that showed BCG to be effective in man were carried out with liquid vaccines, but there is new strong circumstantial evidence that freeze-dried vaccines are similarly effective.

In view of these considerations, a major controlled trial of some of the most frequently used strains of BCG would still be of value, with the aim of re-establishing the properties of seed lots that are suitable for the preparation of a satisfactory freeze-dried BCG vaccine. National laboratories and international organizations should take up this research problem, using vaccines prepared for administration to human beings; vaccines prepared from seed lots concerning the safety and efficacy of which there is little information should be tested in man only with great caution.

Some manufacturers of dried vaccine have modified the strain they use by selecting for constitution a seed lot more suitable for their particular production procedure. Seed lots prepared in this way may not retain their immunogenic properties unchanged, and may be used only after they have been retested by controlled trials in man.

In practice, BCG seed lots may generally be investigated in man only for their properties of producing tuberculin sensitivity and vaccination lesions. The former should be measured by the distribution of tuberculin reactions according to size in persons vaccinated with a given dose of BCG vaccine. A low dose of tuberculin should be employed (e.g., 5 IU of PPD or 2 tuberculin units (TU) of RT23).

Field observations should be made in conjunction with laboratory studies in animals. The latter should include protection tests, tests of vaccination lesions, and tests for tuberculin conversion. Immunizing efficacy should be measured by the degree of protection afforded to the test animals against a challenge with fully virulent Mycobacterium tuberculosis. Sensitizing efficacy should be measured by the average dose of vaccine that will convert a negative tuberculin reaction in guinea-pigs to a positive one, as well as by the speed with which such
conversion is effected. In these animal tests, the inclusion, for comparative purposes, of a reference vaccine prepared from a seed lot known to be effective in animals and man is recommended. Such a reference vaccine is available from WHO.

It is desirable that a proposed replacement international reference preparation should be prepared, because the number of ampoules of the International Reference Preparation still available is limited and also because it has now been stored for almost 20 years. Furthermore, the stock of a "working reference", prepared for laboratory control tests, is almost exhausted.

The selection of the seed lot should be governed by practical criteria relevant to the use of a reference preparation in the laboratory. As with the present International Reference Preparation, the proposed replacement should be suitable for use in man, not only to allow a measure of its performance in man, but also to permit its inclusion in future international field studies.

**POTENCY TESTS**

There is some evidence that BCG seed lots that have been shown to produce vaccines with protective potency in laboratory animals and tuberculin sensitivity in man will give effective protection against tuberculosis in human beings. However, at the present time there is no established laboratory test that can be used in practice to compare the protective efficacy of different vaccines.

The laboratory tests at present in use and included in these requirements are designed to ensure that new vaccine lots do not differ appreciably from those that have already been shown to be effective in man as regards their ability to induce adequate sensitivity to tuberculin, or from a reference vaccine shown to be safe and effective in man. At present, for batch control purposes, much reliance is placed on tests for the estimation of the total bacterial content and number of culturable particles. It is not possible to specify single requirements for the total bacterial content and for the number of culturable particles for all vaccines, since differing seed lots and methods of manufacture may yield differing values for these criteria. In spite of this, however, the vaccines have similar and satisfactory properties as regards their ability to induce adequate sensitivity to tuberculin and their safety in man. It is therefore essential that the national control authorities decide, as a result of extensive
field trials in man, on a suitable range of values for the total bacterial content and for the number of culturable particles for a particular manufacturer's product and for a particular method of manufacture. In addition, it is necessary to perform animal experiments that give an indication of the safety and efficacy of the vaccines to the satisfaction of the national control authority.

POST-VACCINATION REACTIONS AND COMPLICATIONS

A local reaction at the vaccination site is normal after BCG vaccination. It may take the form of a crusted nodule, which in many cases will break down and suppurate. The nodule may persist and ulcerate. Some swelling of regional lymph nodes may also be seen, and this may be regarded as a normal reaction. Keloid may occur at the site of the vaccination scar, and children exhibiting an exaggerated reaction to the vaccine, or keloid formation, should not be revaccinated.

Sometimes an undue proportion of larger reactions, or subcutaneous abscesses, have been seen. This has indicated that the vaccine was not given strictly by the intracutaneous route, and a lack of training of the vaccinator has been suspected. Persistent nodules should not be treated by giving systemic corticosteroids, since a few cases of generalized BCG infection have resulted from such treatment.

Among the major complications, supplicative lymphadenitis has been observed. With certain vaccines, analysis of the incidence of

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1 Numbers of culturable particles in millions/ml for intradermal vaccine used in some countries in adults and children are the following:

<table>
<thead>
<tr>
<th>Country</th>
<th>BCG strain used</th>
<th>Lowest</th>
<th>Highest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>Pasteur</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>Denmark</td>
<td>Danish</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>France</td>
<td>Pasteur</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Germany, Fed. Rep. of</td>
<td>Danish</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Japan</td>
<td>Japanese</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>Netherlands</td>
<td>Pasteur</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>Glaso</td>
<td>8</td>
<td>26</td>
</tr>
<tr>
<td>USSR</td>
<td>USSR</td>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>

It is recommended that the dose for babies should be one-half to one-quarter as much as that for teenage children or adults (6, page 13).
these complications in the newborn has revealed a strong correlation with the number of culturable particles. Reduction of the dose for the newborn may reduce these complications to acceptable levels. Therefore, it is required that the concentration of the vaccine should be shown to be effective and tolerated in the age groups for which the vaccine is intended.

Rare cases of osteitis after vaccination in the newborn have been reported from parts of northern Europe, with onset at 6–36 months of age. All cases occurred after vaccination into the thigh, with an estimated frequency of between 1 in 10,000 and 1 in 100,000 babies vaccinated.

PRESENTATION AND USE OF THE REQUIREMENTS

Each of the following sections constitutes a recommendation. The parts of each section that are printed in type of normal size have been written in the form of requirements, so that, if a health administration so desires, these parts as they appear may be used as definitive national requirements. The parts of each section that are printed in small type concern points on which comments seem desirable.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning BCG vaccine, it is recommended that a clause should be included permitting modifications of the manufacturing requirements on condition that such modified requirements ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements formulated below. The World Health Organization should then be informed of the action taken.

The terms “national control authority” and “national control laboratory”, as used in these requirements, always refer to the country in which the vaccine was manufactured.

PART A. MANUFACTURING REQUIREMENTS

1. DEFINITION

1.1 International name and proper name

The international name shall be “Vaccinum Tuberculosis (BCG) Cryodesiccatum”. The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.
1.2 Descriptive definition

Vaccinum Tuberculosis (BCG) Cryodesiccatum is a dried preparation containing live bacteria derived from a culture of the bacillus of Calmette and Guérin, known as BCG, intended for intradermal injection. The preparation shall satisfy all the requirements formulated below. In the case of dried vaccine intended for percutaneous vaccination, the name is “Vaccinum Tuberculosis (BCG) Cryodesiccatum Percutaneum”.

Vaccinum Tuberculosis (BCG) Cryodesiccatum is referred to in this document as “dried BCG vaccine”.

1.3 International standard, international reference preparations, and international units

The International Reference Preparation of BCG vaccine (established in 1965) is dispensed in ampoules containing dried BCG vaccine derived from 2.5 mg (semi-dry weight, as defined in 7) of bacillary mass of BCG and 5 mg of sodium glutamate (total weight of dried material: 5.72 mg per ampoule).

The International Standard for Purified Protein Derivative (PPD) of Mammalian Tuberculin (established in 1951) is dispensed in ampoules containing 10 mg of PPD plus 4 mg of salts. The International Unit of PPD (mammalian) is defined as the activity contained in 0.000025 mg of the International Standard.

A reference preparation of opacity may be used during the preparation of BCG vaccine in estimating its total bacterial content. The fifth International Reference Preparation of Opacity (established in 1975) consists of a plastic rod simulating the optical properties of a bacterial suspension (10 IU of opacity). It is intended for visual comparison.

Since the relationship of opacity to bacterial mass in BCG vaccine is dependent on the method of preparation of the vaccine, it is necessary to establish for any one manufacturer’s vaccines and for a particular method of manufacture the relationship between the total bacterial content of the vaccines and their opacity, as determined in relation to the International Reference Preparation of Opacity.

The above-mentioned International Standard of Tuberculin and the International Reference Preparation of BCG vaccine are in the custody of the International Laboratory for Biological Standards, State Serum Institute, Copenhagen, and samples are distributed free of charge on request to national control laboratories. The international standard and international reference preparation are intended for the calibration of national standards for tuberculin and national reference preparations of
BCG vaccine. The International Reference Preparation of Opacity is in the custody of the International Laboratory for Biological Standards, London, which supplies samples on request. Such preparations should be used in the laboratory control of potency of tuberculins intended for testing skin sensitivity in man and animals and in the production and control of BCG vaccine.

1.4 Terminology

Seed lot. A quantity of bacteria processed together and of uniform composition. A seed lot shall be maintained in the dried form. In each manufacturing establishment, a primary seed lot is that from which material is drawn for inoculating media for the preparation of secondary seed lots or single harvests.

Secondary seed lot. A working seed lot, not more than four culture passages removed from the primary seed lot, having the same characteristics of the primary seed lot and intended for inoculating media for the preparation of single harvests.

Single harvests. A quantity of bacteria harvested from cultures prepared in a single batch of medium and processed together. Single harvests shall be prepared from cultures removed from a primary seed lot by as few cultural passages as possible, and by not more than 12 passages.

Final bulk. The homogeneous finished vaccine present in a single container. It may then be filled either directly or through one or more intermediate containers.

Vaccine lot (vaccine batch). Vaccine in final containers from a single final bulk subsequently processed together and that have, therefore, a uniform composition before drying.

Filling lot. A collection of filled final containers that are homogeneous with respect to the risk of contamination during filling.

Final lot. A final lot is a collection of final containers from one filling lot, which have been dried together in the same vacuum chamber.

2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General
Requirements for Manufacturing Establishments and Control Laboratories (8, page 13) shall apply to establishments manufacturing BCG vaccine, with the addition of the following.

Production of BCG vaccine shall take place in completely separate areas, by means of separate equipment. Such areas shall be so situated and ventilated that the hazard of contamination is reduced to a minimum. No animals shall be permitted in the vaccine production areas. Tests necessary for the control of the vaccine that may result in the culture of contaminating microorganisms, or in which animals are used, shall be carried out in a topographically separate area.

For the purposes of these requirements, the processes of vaccine production that must take place in isolated facilities are all operations up to and including the sealing of the vaccine in the ampoules. The packaging may be done in a common area.

In some countries, the production of BCG vaccine—although isolated—is carried out in a building in which other work takes place. This should be done only after consultation with, and with the approval of, the national control authority. If production takes place in part of a building, the work carried on in the other part of the building should not be of such a nature that there is any possibility of hazard to the BCG vaccine.

During production, no cultures of microorganisms other than strains of BCG approved by the national control authority for vaccine production shall be introduced into the manufacturing areas. In particular, no strains of other mycobacterial species, whether pathogenic or not, shall be permitted in the BCG vaccine production area.

BCG is susceptible to sunlight. Therefore, the procedures for the preparation of the vaccine shall be so designed that all cultures and vaccines are protected from the effects of daylight and ultraviolet light at all stages of manufacture, testing, and storage, until the vaccine is issued.

BCG vaccine shall be produced by a staff consisting of healthy persons who shall not work with other infectious agents; in particular, they shall not work with virulent strains of M. tuberculosis, nor shall they be exposed to a known risk of tuberculosis infection. Precautions shall be taken also to ensure that no worker shall be employed in the preparation of BCG vaccine unless he or she has been shown by medical examination to be free from tuberculosis. The scope and nature of the medical examination shall be at the
discretion of the national control authority, but it shall include a radiological examination and shall be repeated at intervals or when there is cause to suspect illness.

The frequency with which the radiological examination should be carried out is at the discretion of the national control authority. It is advisable to keep radiation exposure to a minimum, but the examination should be of sufficient frequency to detect the appearance of early active tuberculosis. It is estimated that, if workers in BCG vaccine laboratories were given one or two conventional X-ray examinations of the chest each year, not using fluoroscopic methods, and if the best available techniques were employed to minimize the radiation dose, the doses received would be considerably lower than the maximum permissible doses for workers occupationally exposed to radiation that have been set by the International Commission on Radiological Protection (9, 10).

Should an examination reveal signs of tuberculosis or suspected tuberculosis in a worker, he or she shall no longer be allowed to work in the production areas and the rest of the staff shall be examined for possible tuberculous infection. In addition, all cultures\(^2\) shall be discarded and the production areas decontaminated. If it is confirmed that the worker has tuberculosis, all vaccine made while he or she was in the production areas shall be discarded.

Suitable laboratory clothing shall be worn in the production areas.

Persons not normally employed in the production areas shall be excluded from them unless, after a medical examination, including radiological examination, they are shown to be free from tuberculosis. In particular, persons working with mycobacteria other than the BCG seed strain shall be excluded at all times.

Written descriptions of procedures for the preparation of BCG vaccine shall be submitted for approval to the national control authority. Proposals for modification shall be submitted for approval to the national control authority before their implementation.

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\(^2\) In this respect, the freeze-dried seed, which is in ampoules sealed by the fusion of glass, is not regarded as a culture.
3. PRODUCTION CONTROL

3.1 Control of source materials

3.1.1 Seed lot system

The production of vaccine shall be based on the seed lot system. A seed lot prepared from a strain approved by the national control authority (see Part B, section 1.1) shall be prepared under conditions satisfying the requirements of Part A, sections 2 and 3.

A seed lot should have been shown in field trials in man to induce adequate sensitivity to tuberculin and to be safe, and also have been tested for ability to produce protection against tuberculous infection in animals.

It is recommended that a large primary seed lot should be set aside under optimum storage conditions as the basic material to which the manufacturer may return for the preparation of further seed lots. This primary seed lot may be part of a vaccine lot that has been shown in extensive field trials in man to induce adequate sensitivity to tuberculin, with only a low frequency of untoward effects, and in laboratory experiments in animals to protect against tuberculous infection.

3.1.2 Tests on seed lot

The seed lot used for the production of vaccine shall be tested according to the requirements of Part A, sections 5.1, 5.2, and 5.3. However, the test for absence of virulent mycobacteria, described in Part A, section 3.4.3, shall be made in at least 10 guinea-pigs. The dose of seed lot to be used in this test shall contain a number of culturable particles equivalent to that injected when a vaccine lot is tested. The guinea-pigs shall be observed for at least 6 months. At the end of the observation period, the animals shall be killed and examined post mortem for macroscopic evidence of progressive tuberculous disease; similarly, any animals that die before the end of the observation period shall be subjected to post-mortem examination. If none of the animals shows signs of tuberculosis and at least 60% of them survive the observation period, the seed lot shall be considered to be free from virulent mycobacteria.

In addition, it is advisable to test all primary seed lots for protective potency in animals. In such tests, the material, when injected into guinea-pigs and mice in a range of doses under reproducible conditions, should afford a measure of protection, against a range of challenge doses of a fully virulent human
strain of \textit{M. tuberculosis}, not less than that specified by the national control authority in terms of a national reference preparation such as the one described in Part A, section 1.3.

In some countries, an additional test is included to ensure that the first batch of vaccine produced from a new ampoule of the seed lot gives rise to tuberculin sensitivity in guinea-pigs. It is desirable also that each seed lot should be tested to demonstrate that its degree of sensitivity to antituberculosis drugs has not changed.

3.1.3 \textit{Production culture medium}

The production culture medium shall contain no substances unsuitable for injection into human beings. Substances used in that medium shall meet such specifications as the national control authority may prescribe.

3.2 \textit{Production precautions}

The general precautions formulated in the requirements of Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) \((8,\ page\ 15)\) shall apply to the manufacture of BCG vaccine, with the addition of the following.

Special precautions shall be taken to prevent the accidental mixing of containers from separate lots of vaccine at all stages of production up to the stage at which final lots are labelled.

3.3 \textit{Control of single harvests}

The cultures in each container shall be examined visually, and any that have grown in an uncharacteristic manner shall not be used for vaccine production.

3.4 \textit{Control of final bulk}

3.4.1 \textit{Final bulk}

The final bulk shall be prepared from a single harvest or by pooling a number of single harvests.
3.4.2 Test for absence of contaminating microorganisms

The final bulk shall be tested for bacterial and mycotic contamination in accordance with the requirements given in Part A, section 5, of Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (11, page 48), and no vaccine lot shall be passed for use unless the final bulk has been shown to be free from such contamination.

3.4.3 Test for absence of virulent mycobacteria

A sample of the final bulk intended for this test shall be stored for not more than 72 hours after harvest at 4 °C.

At least 6 guinea-pigs, all of the same sex, each weighing 250-350 g are used.

Some countries prefer to use guinea-pigs that have been shown previously to be tuberculin-negative.

A dose of BCG organisms corresponding to at least 50 human doses of vaccine intended for intradermal injection shall be injected into each guinea-pig by the subcutaneous or intramuscular route. The animals shall have been maintained on a diet that is free from added substances, such as antibiotics, that might interfere with the test. The guinea-pigs shall be observed for at least 6 weeks. If, during that time, they remain healthy and gain weight, the final bulk shall be considered to be free from virulent mycobacteria.

At the end of the observation period, the animals shall be killed and examined post mortem for macroscopic evidence of progressive tuberculous disease; similarly, any animals that die before the end of the observation period shall be subjected to post-mortem examination.

The vaccine lot shall pass the test if none of the guinea-pigs shows evidence of progressive tuberculous disease and if at least two-thirds of them survive the observation period.

Should more than one-third of the guinea-pigs die during the observation period (and freedom from progressive tuberculous

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3 When a more concentrated vaccine, intended for administration by the percutaneous route, is tested, a dilution factor approved by the national control authority shall be applied, so that the mass of BCG injected corresponds to 50 human doses of intradermal vaccine.
disease is verified), the test shall be repeated with another sample of the final lot on at least 6 more guinea-pigs.

If, on the second occasion, more than one-third of the animals fail to survive the observation period, the vaccine lot shall not pass the test.

Should a vaccine lot fail to satisfy the requirements of this test because animals die from causes other than tuberculosis, the procedure to be followed by the manufacturer should be determined with the approval of the national control authority.

If evidence of progressive tuberculous disease is seen, the vaccine lot shall be rejected, all subsequent vaccine lots shall be withheld, and all current vaccine stocks shall be held pending further investigation. The manufacture of BCG vaccine shall be discontinued and it shall not be resumed until a thorough investigation has been made and the cause or causes of the failure determined, and then only with the approval of the national control authority.

3.4.4 Test for bacterial concentration

The bacterial concentration of the final bulk shall be estimated by a method approved by the national control authority and shall have a value within a range approved by the national control authority (see Part B, section 1.2).

The estimation of total bacterial content may be made either directly, by determining the dry weight of organisms, or indirectly, by an opacity method that has been calibrated in relation to the dry weight of organisms.

It is desirable that one method of estimation should be adhered to for all the vaccine lots produced by a manufacturer.

If an opacity method is used, the International Opacity Reference Preparation, or an equivalent reference preparation approved by the national control authority, may be employed in comparative tests.

3.4.5 Test for number of culturable particles

The number of culturable particles of each final bulk shall be determined by an appropriate method approved by the national control authority.

The medium used in this test should be such that the number of culturable particles may be determined not more than 4 weeks
after the medium has been inoculated with dilutions of vaccine.
There are various methods of determining the number of culturable particles in BCG vaccine, and it is desirable that one method should be adhered to for all the vaccine lots produced by a manufacturer (7). It is also desirable that the test should be carried out in parallel with a reference preparation.

3.4.6 *Oxygen uptake test*

Some manufacturers use a measurement of oxygen uptake rate (8, page 16) as a speedy test that may be carried out on the final bulk vaccine, before filling, to give some indication of the viability of the batch. Usually 30–120 mg (semi-dry weight) are tested in a Warburg apparatus, and the respiration is measured over an hour or more.

3.4.7 *Substances added to the final bulk*

Substances used in preparing the final bulk, or added thereto, shall meet such specifications as the national control authority may prescribe. Substances added to improve the efficiency of the freeze-drying process or to aid stability of the dried product shall be sterile and of high and consistent quality, and shall be used at suitable concentrations in the vaccine.

4. **FILLING AND CONTAINERS**

The requirements concerning filling and containers given in Part A, section 4, of the Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (8, page 16) shall apply, with the addition of the following.

It shall have been demonstrated to the satisfaction of the national control authority that the containers are made from materials that have no deleterious effect on the vaccine.

The containers should be in a form that renders the process of reconstitution as simple as possible. Their packing should be such that the reconstituted vaccine is protected from daylight. It is desirable that the capacity of the final containers should be large enough for the full volume of the reconstituting fluid to be added.
Single-dose and multiple-dose containers may be used; the latter should be of a type that will not encourage storage of the reconstituted vaccine.

All containers of the final vaccine shall be sterilized before filling.

Provision shall be made for adequate and continuous mixing of the final bulk during filling into final containers.

As soon as possible after harvesting, the vaccine shall be filled into containers and freeze-dried.

Containers shall be hermetically sealed, either under vacuum or after filling with pure (especially oxygen-free), dry nitrogen or any gas not deleterious to the vaccine. It shall be shown to the satisfaction of the national control authority that the vaccines stored under a gas have satisfactory stability.

All containers sealed under vacuum shall be tested for leaks not less than 2 months after sealing, and all defective containers shall be discarded.

In some countries, the test for leaks is repeated after further storage. The most convenient time to do this is immediately before distribution.

5. CONTROL TESTS ON FINAL LOT

Tests on the final lot shall be performed after it has been reconstituted. The fluid supplied or recommended for reconstitution shall be used, unless such fluid would interfere with any of the tests, in which case some other suitable fluid shall be used. The vaccine shall be reconstituted to the concentration at which it is to be used for injection into human beings.

An exception may be made in the case of the test for absence of virulent mycobacteria (Part A, section 5.3.1), when a higher concentration of reconstituted vaccine may be necessary.

5.1 Identity test

An identity test shall be performed on samples of vaccine from each final lot.

The identity of each filling lot of vaccine shall be verified by the morphological appearance of the bacilli in stained smears and by the characteristic appearance of the colonies grown on solid media.
5.2 Test for absence of contaminating microorganisms

Samples from each final lot shall be tested for bacterial and mycotic contamination according to the requirements given in Part A, section 5, of Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances) (II, page 48).

5.3 Safety tests

5.3.1 Test for absence of virulent mycobacteria

If the test for the absence of virulent mycobacteria, applied to the final bulk, is unsatisfactory, it shall be repeated with a sample of a final lot (see Part A, section 3.4.3).

This test may be applied to the final lot and omitted from the final bulk.

5.3.2 Test of skin reactivity in guinea-pigs

Some manufacturers include a test of the reactivity of each vaccine lot when injected intradermally into guinea-pigs. The injections consist of 0.1 ml of undiluted vaccine 1 and 0.1 ml each of vaccine dilutions 1:10 and 1:100. At least 4 guinea-pigs are used, all of the same sex and weighing not less than 300 g each. If female guinea-pigs are used, they should not be pregnant.

The lesions produced are observed for at least 4 weeks. The reactions caused should be compared with a reference preparation approved by the national control authority and that has been shown to induce in man adequate sensitivity to tuberculin with a low frequency of untoward effects.

The same dilutions of the reference preparation and vaccine should be injected into the same guinea-pigs at randomly selected sites.

5.4 Test for total bacterial content

The total bacterial content of the reconstituted vaccine shall be estimated for each vaccine lot by a method approved by the national control authority, and shall have a value within a range approved by the national control authority (see Part B, section 1.2).

The estimation of total bacterial content may be made either directly, by determining the dry weight of organisms, or

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1 Percutaneous vaccine should be diluted (see Part A, section 3.4.3).
indirectly, by an opacity method that has been calibrated in relation to the dry weight of organisms. It is desirable that one method of estimation should be adhered to for all the vaccine lots produced by a manufacturer.

5.5 Test for number of culturable particles

The number of culturable particles of each final lot shall be determined by an appropriate method approved by the national control authority (see Part A, section 3.4.5). The viable count shall have a value within a range approved by the national control authority (see Part B, section 1.2). By comparison with the results of the test for number of culturable particles carried out on final bulk, as described in Part A, section 3.4.5, the percentage survival on freeze-drying may be calculated and this value should be not less than one approved by the national control authority.

The survival rate usually varies between 75% and 20%, although a survival rate as low as 10% is permitted by some national control authorities.

5.6 Stability test

Each final lot shall be tested for its degree of stability by a method approved by the national control authority.

The object of this test is to obtain experimental evidence on which to base the statements concerning storage temperature and expiry dates that appear on the label and the leaflet as required in Part A, section 8.

The test shall involve the determination of the number of culturable particles before and after the samples have been held at appropriate temperatures and for appropriate periods.

An accelerated degradation test shall be carried out by taking samples of the vaccine and incubating them at 37°C for 28 days. The percentage fall in the number of culturable particles is then compared with that of the same vaccine stored at 4°C. The number of culturable particles in the vaccine after heating shall be not less than 20% of that in the refrigerated vaccine. The absolute value shall be approved by the national control laboratory. The test shall also be made in parallel with a reference preparation. One method of determining the number of culturable particles shall be adhered to, as suggested in Part A, section 3.4.5.
The purpose of including a reference preparation is to have a check on the quality of the medium used for the determination of the number of cultivable particles. It is not intended to adjust the count of the vaccine by comparison with the reference preparation.

All manufacturers shall keep their product for the permitted storage period and shall determine the number of cultivable particles to demonstrate that this has been maintained at an adequate level.

In some countries, the stability test is carried out only after the vaccine has been stored for 3–4 weeks after drying, since it is considered that the degree of stability during the first 3 weeks may not be related to the long-term stability of the product. As a guide to stability, some manufacturers of dried BCG vaccine determine the residual water content of the final vaccine, since failure to achieve a certain degree of desiccation results in an unstable product. However, such a test cannot be regarded as an alternative to tests involving the determination of the number of cultivable particles.

5.7 Production consistency

The manufacturer shall show to the satisfaction of the national control authority that the method of manufacture ensures a consistent product.

The degree of consistency in producing satisfactory final lots is an important factor in judging the efficacy and safety of a particular manufacturer’s product.

The data that shall be considered in determining the consistency of production shall include the results obtained with consecutive vaccine lots when tested as described in Part A, section 5, for example, by the test for total bacterial content (Part A, section 5.4), the test for number of cultivable particles (Part A, section 5.5), and the stability test (Part A, section 5.6).

In addition, the examination of morphological appearance of the bacteria in stained smears (Part A, section 5.1) or in unstained microscopic preparations may be used to determine the consistency of bacterial aggregation.

A specified number of consecutive vaccine lots shall have been satisfactorily prepared before any vaccine from a given manufacturer, or resulting from a new method of manufacture, is released. In subsequent routine production, if a specified proportion of vaccine lots or a specified number of consecutive vaccine lots fails
to meet the requirements, the manufacture of BCG vaccine shall be discontinued and not be resumed until a thorough investigation has been made and the cause or causes of the failures determined to the satisfaction of the national control authority.

6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (§ page 17) shall apply, with the addition of the following.

Written records shall be kept of all seed lots, all cultures intended for vaccine production, all single harvests, all final bulk vaccines, and all vaccine in the final containers produced by the manufacturing establishments, including all tests irrespective of their results.

The records shall be of a type approved by the national control authority. An example of a suitable protocol is to be found on pages 141–145.

7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (§ page 18) shall apply.

It is desirable that samples should be retained for at least one year after the expiry date for all the vaccine in the final lot.

8. LABELLING

The requirements given in Part A, section 8 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (§ page 18) shall apply, with the addition of the following.

The label printed on or affixed to each container shall show:
— the volume and nature of the reconstituting fluid.
Moreover, this label, or the label on the carton enclosing several final containers, or the leaflet accompanying the containers, shall contain the following additional information:
— The fact that the vaccine fulfills the requirements of this document;
— instructions for use of the vaccine and information concerning contra-indications and the reactions that may follow vaccination;
— the conditions recommended during storage and transport, with information on the reduced stability of the vaccine if exposed to temperatures higher than that stated on the label;
— a warning that the vaccine must be protected from daylight both before and after reconstitution; and
— a statement that, after a final container has been opened and its contents reconstituted, the vaccine must be used as soon as possible and, in any event, within 2 hours, and that any portion not used within this period should be discarded.

9. DISTRIBUTION AND TRANSPORT

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 18) shall apply.

It is the responsibility of the manufacturer to use means of transport likely to maintain the potency of the vaccine.

10. STORAGE AND EXPIRY DATE

The statement concerning storage temperature and expiry dates, appearing on the label and leaflet as required in Part A, section 8, shall be based on experimental evidence obtained from the tests indicated in Part A, section 5.6, and shall be submitted for approval to the national control authority.

10.1 Storage conditions

Before being distributed by the manufacturing establishment, or before being issued from a depot for the storage of vaccine, all
vaccines in their final containers shall be kept constantly at a temperature below 6 °C and shall be protected from daylight.

Precautions should also be taken to maintain the vaccine, during transport and up to the time of use, at the temperature and under the storage conditions recommended by the manufacturer.

10.2 Expiry date

The date after which dried BCG vaccine may not be used shall be determined in relation to the experimental evidence referred to in Part A, section 5.6, and with the approval of the national control authority. The date shall be not more than 12 months after the date of issue, provided that this is not more than 24 months from the date of the last satisfactory test for culturable particles, referred to in Part A, section 5.5, and provided that the vaccine has been stored continuously at the specified storage temperature and protected from daylight. In any event, the expiry date shall be not more than 3 years from the date of harvest. Each manufacturer shall test the stability of the vaccine to ensure that it satisfies these conditions.

PART B. NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories, given in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (8, page 19), shall apply.

In addition, the national control authority shall provide a reference vaccine (see Part A, section 1.3) or approve one used by a manufacturer, and shall give directions concerning the use of the reference vaccine in specified tests. The national control authority shall also give directions to manufacturers concerning the BCG strain to be used in vaccine production, the total quantity of bacteria, the number of culturable particles, and the stability required of the vaccine, and shall specify the requirements to be fulfilled by the manufacturer in accordance with the provisions of Part A of this document, including those for consistency of quality in respect of the points referred to in Part A, section 5.7.
1.1 BCG strain

The strain of BCG (maintained in the form of a seed lot) used in the production of vaccine shall be derived from the original strain maintained by Calmette and Guérin and shall be identified by historical records. On the basis of cultures and biochemical and animal tests,¹ the BCG seed lot shall show characteristics that conform to those of BCG and generally differ from those of other mycobacteria. The seed lot shall show consistency of the morphological appearance of colonies on serial subculture. It shall also have been shown to yield vaccines that, upon administration by intradermal injection to children and adults, induce adequate sensitivity to tuberculin, with the occurrence of a low frequency of untoward effects. In addition, the seed lot shall have been shown to give adequate protection against tuberculosis in experimental animals in tests for protective potency such as are referred to in Part A, section 3.1.2. It is advisable also to investigate the duration of the tuberculin sensitivity produced by the seed lot.

1.2 Concentration of BCG vaccine

The concentration of BCG vaccine varies with different vaccines and is dependent on a number of factors, such as the strain of BCG used and the method of manufacture. It is therefore essential, for each manufacturer as well as for each different method of manufacture, for the optimum potency of vaccine to be ascertained by trials in tuberculin-negative subjects (new-born babies, older children, and adults) to determine the response to vaccination in respect of the induction of adequate sensitivity to tuberculin, the production of satisfactory local skin lesions, and the occurrence of a low frequency of untoward reactions. As a result of such trials, the national control authority shall give directions to the manufacturer concerning the total bacterial content and the number of culturable particles required of the vaccine.

If a manufacturer changes his procedure of preparing BCG vaccine, and if the national control authority considers that the change might affect the final product, it may be necessary to conduct further trials in order to determine the optimum content of BCG organisms in the new product.

¹ In investigating various BCG strains, it may be advisable to test their infectivity in animals, including hamsters.
2. RELEASE AND CERTIFICATION

A lot of dried BCG vaccine shall be released only if it fulfils Part A of the present requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify that the lot of vaccine in question meets all national requirements as well as Part A of the present requirements. The certificate shall also state the date of the last satisfactory test for number of culturable particles, the expiry date, the lot number, the number under which the lot was released, and the number appearing on the labels of the container. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of dried BCG vaccine between countries.

3. CLINICAL SURVEILLANCE OF THE VACCINE IN MAN

The national control authorities shall satisfy themselves that adequate control of BCG vaccine has been achieved, by arranging for studies in children to be made at regular intervals on some of the final lots prepared.

Such studies should be made, for each vaccine lot tested, in at least 100 tuberculin-negative persons, and records should be obtained of the degree of sensitivity to tuberculin induced (distribution of tuberculin reactions by size) with a defined low dose of tuberculin, local skin lesions (nature and size of BCG vaccine reaction), and the occurrence of untoward vaccination reactions. It is desirable that such tests should be performed in

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6 Where a manufacturer has no national control authority, advice and help may be requested from: Chief, Biologics, WHO, Geneva.

7 In some countries, the proportion of cases showing a negative reaction to tuberculin before BCG vaccination, but giving a positive result after vaccination, is called the “tuberculin conversion rate”. Unless positive and negative reactions are carefully defined, however, such a rate may not reveal certain cases in which a weak reaction is changed after BCG vaccination into a strong reaction to a low dose of tuberculin.

8 An intradermal test with a dose of tuberculin equivalent to 5 IU of tuberculin PPD is suitable. A description of an appropriate method is obtainable on application to Chief, Biologics, WHO, Geneva.

9 A design for a study to assess BCG vaccines in man is available on application to Chief, Biologics, WHO, Geneva.
parallel on two or more vaccine lots in the same population group, one of the vaccine lots being preferably a reference vaccine.

The frequency of testing batches will depend on the number of batches of vaccines produced, but, in any case, at least one of the batches produced each year should be tested. The age group vaccinated should be that in which the vaccine will be used.

**SUMMARY PROTOCOL FOR DRIED BCG VACCINE PRODUCTION AND TESTING**

(Based on the Requirements for Biological Substances, No. 11—

**Identification of Final Lot**

<table>
<thead>
<tr>
<th>Name and address of manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot No. and type of vaccine</td>
</tr>
<tr>
<td>Date of manufacture of final lot</td>
</tr>
</tbody>
</table>

**Information on Seed Lot**

1. **Seed lot**

   Identity of seed lot
   Origin of seed lot
   Date of preparation of seed lot
   Date of reconstitution of seed lot
   Date of receipt of seed lot

2. **Tests on seed lot** (If these data on the same seed lot have been submitted before, completion of this paragraph is not necessary.)

   (a) **Identity test**
       Identified as BCG yes/no

   (b) **Test for absence of contaminating microorganisms**
       Date of start of test
### Date of end of test

Results

<table>
<thead>
<tr>
<th>(c) Safety test</th>
<th>First test</th>
<th>Repeat test if necessary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of virulent mycobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose injected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of guinea-pigs given injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight range and sex of guinea-pigs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observation period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Health of animals during test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight gain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Results (Passed/Failed)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Seed lot approved  yes/no

Date of approval  

**Information on Manufacture**

3. Single harvest

| No. of passages from seed |            |                          |
| Medium |            |                          |
| No. of containers inoculated |            |                          |
| Date of inoculation |            |                          |
| Date of harvest |            |                          |
| Visual inspection and results |            |                          |

4. Final bulk

| Date of preparation |            |                          |
| No. of single harvests included |            |                          |

(a) Absence of contamination

| Quantity tested |            |                          |
| Media |            |                          |

Duration of test  

---
### Results

(b) *Absence of virulent mycobacteria*

<table>
<thead>
<tr>
<th>First test</th>
<th>Repeat test if necessary</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of human doses injected</td>
<td></td>
</tr>
<tr>
<td>No. of guinea-pigs given injection</td>
<td></td>
</tr>
<tr>
<td>Weight range and sex of guinea-pigs</td>
<td></td>
</tr>
<tr>
<td>Observation period</td>
<td></td>
</tr>
<tr>
<td>Health of animals during test</td>
<td></td>
</tr>
<tr>
<td>Weight gain</td>
<td></td>
</tr>
<tr>
<td>Results (Passed/Failed)</td>
<td></td>
</tr>
</tbody>
</table>

(c) *Substances added to final bulk and concentration*

5. *Freeze-drying*

| Type and size of containers |  |
| No. of doses per container |  |
| Method used for sealing the containers | Vacuum-sealing/Sealing under gas |
| No. of containers of each size in the final lot |  |

### Information on Final Product Controls

Recommended reconstitution fluid

Volume of reconstitution fluid per final container

6. *Identity test*

| Type of test |  |
| Results |  |

7. *Absence of contamination*

<table>
<thead>
<tr>
<th>First test</th>
<th>Repeat test if necessary</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of containers tested</td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td></td>
</tr>
</tbody>
</table>
### 8. Safety tests

#### (a) Absence of virulent mycobacteria (if test not performed on final bulk)

<table>
<thead>
<tr>
<th></th>
<th>First test</th>
<th>Repeat test if necessary</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of human doses injected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution factor applied if percutaneous vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of guinea-pigs given injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight range and sex of guinea-pigs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observation period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Health of animals during test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight gain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Results (Passed/Failed)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### (b) Skin reactivity test (if performed)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Dilution injected</th>
<th>Mean diameter</th>
<th>Reference</th>
<th>Dilution injected</th>
<th>Mean diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Period of test | | | |
|----------------|---|-------------|
|                 |   |             |

#### (c) Total bacterial content

<table>
<thead>
<tr>
<th>Method of estimation</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Result (content of semi-dry organisms in mg/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### (d) Culturable particles

<table>
<thead>
<tr>
<th>Method of determination</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Medium
No. of containers tested
Mean count of culturable particles per ml and standard deviation
Date of start of test

9. Stability test
Date of start of test
Temperature of incubation
Time of incubation
No. of containers tested
Mean percentage of survival

Information on Release
Is the vaccine satisfactory? yes/no
Has the lot been released by the national control authority? yes/no
If yes, date
Can a certificate be supplied by the national control laboratory? yes/no
Which laboratory would supply such a certificate?
When was the vaccine tested in children in your country? (summary of results)

Signature of head of laboratory

Certification by person taking overall responsibility for production of the vaccine:
I certify that lot No. of BCG vaccine satisfies Part A of the WHO Requirements for BCG Vaccine.

Signature
Name typed

The protocol must be accompanied by a sample of the label, a copy of the leaflet, and a copy of the national control release certificate, if issued.
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REFERENCES

REVISED REQUIREMENTS FOR INFLUENZA VACCINE (INACTIVATED)
(Requirements for Biological Substances No. 17)
(Revised 1978)

REQUIREMENTS FOR INFLUENZA VACCINE (LIVE)
(Requirements for Biological Substances No. 28)

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REVISED REQUIREMENTS FOR INFLUENZA VACCINE (INACTIVATED)

(Requirements for Biological Substances No. 17)
(Revised)

INTRODUCTION

The only generally accepted means of influenza prophylaxis available at present is vaccination. The various types of vaccination procedure, with live attenuated or killed vaccines, have been reviewed by a WHO Scientific Group on Human Viral and Rickettsial Vaccines (1), which found that killed vaccines are preferred in most countries.

Since influenza vaccines were in use in many countries on a wide scale, in 1967 a group of experts formulated requirements for inactivated influenza vaccine and these were published as an annex to the twentieth report of the Expert Committee on Biological Standardization (2). During the next 5 years, technical developments in
the purification of the virus suspensions from which vaccines were made, as well as in the measurements of the virus content, were such that the potency of whole virus vaccines could be expressed in international units. Accordingly, an addendum to the requirements was annexed to the twenty-fifth report of the Expert Committee on Biological Standardization (3, pp. 15–17). Since then, however, technical developments have completely altered the method of measurement of the haemagglutinin content of the vaccines.

The WHO Expert Committee on Biological Standardization, in its twenty-ninth report (4), recognized that the International Reference Preparation of Influenza Virus Haemagglutinin (Type A) established in 1967 was no longer appropriate for the measurement of haemagglutinin content of virus strains appearing more recently. Accordingly the International Reference Preparation was withdrawn and, furthermore, the Committee recommended that the requirements for inactivated influenza vaccine should be completely revised.

The revised international requirements for inactivated influenza vaccines have been fitted into the framework adopted in the Requirements for Biological Substances No. 1–27, already published by WHO (4, page 142). A group of WHO consultants and staff members (listed on page 192) drafted them, taking into account the regulations and requirements for the manufacture and control of inactivated influenza vaccine in a number of countries, as well as information from published and unpublished reports. In addition, opinions and data have been received from a number of experts, whose assistance is acknowledged (see page 192).

GENERAL CONSIDERATIONS

Inactivated influenza vaccines have been in widespread use for about 35 years. The efficacy of immunization has varied according to circumstances, but protection rates of 75–90% have been reported. Differences in protective efficacy may result from continuing antigenic variation in the prevailing viruses.

Because of such variation the composition of inactivated influenza virus vaccine, unlike that of most viral vaccines, must be kept constantly under review. Accordingly, WHO publishes annually recommendations concerning the strains to be included in the vaccine.
When a new subtype of influenza A virus bearing new haemagglutinin (and neuraminidase) antigen(s) appears, it is likely that vaccine containing the antigens of the formerly prevalent influenza A subtype will be ineffective, and a vaccine containing the new pandemic virus will be required. In addition, during interpandemic periods, influenza A viruses undergo frequent and progressive antigenic drift in their haemagglutinin and neuraminidase antigens. Vaccines containing formerly prevalent viruses are expected to be less protective against virus variants showing antigenic "drift" than against the homologous virus.

Influenza B strains do not vary in subtype. They undergo antigenic variation but with much less frequency than the A strains. Therefore, the influenza B component of bivalent vaccines requires less frequent change. Influenza vaccines usually contain one or more influenza A viruses of a single subtype and one influenza B virus. Occasionally, as in 1977–78, two influenza A subtypes may circulate concurrently. Vaccines containing both subtypes will then be required.

As a result of developments in technology, the WHO Expert Committee on Biological Standardization, in its twenty-ninth report (4), recommended that the potency of influenza vaccines should be expressed in micrograms of haemagglutinin per ml (or dose) as determined by suitable immunodiffusion methods. Appropriate techniques include single-radial-immunodiffusion and Laurell rocket immunoelectrophoresis. In order to standardize the methods, reference antigen (calibrated in micrograms of haemagglutinin per ml) and specific antihemagglutinin serum, suitable for use in the assay of the haemagglutinin content of each component of inactivated vaccines, are prepared and distributed by the National Institute for Biological Standards and Control, London. A new reference antigen and antiserum will be prepared each time it is necessary to introduce a new virus strain into the vaccines.

The formulation of a permanent and inflexible recommendation for the quantity of antigen of the A and B components of inactivated influenza vaccines is inappropriate. This is because of the antigenic variability of the viruses and the fact that, for new variants, differing amounts of antigen will be required to induce antibody levels consistent with immunity. The antibody response depends on the age of the vaccinated person, the presence or absence of prior immunological experience with viruses possessing haemagglutinin (and neuraminidase) antigens related to that of the vaccine strain, and the
number of doses and type of vaccine given (whole virus, disrupted virus antigen, purified surface antigen vaccines). Vaccine potency should be based on the results of clinical trials with various types of vaccine, performed at appropriate times, but particularly on the emergence of new variant strains in various age groups. The trials should include studies of antibody responses to vaccination, clinical reactions, and, where possible, the protective efficacy of the vaccine against infection by natural or artificial challenge.

Recent extensive clinical studies have provided a greater understanding of the parameters affecting the antibody responses and reactions to inactivated influenza vaccines in various age groups. Reactions, particularly in unprimed children, were noted mainly when whole-virus vaccines were given, whereas disruption of the virus decreased the reactogenicity. However, disruption of virus particles also diminished the ability of the vaccine to elicit antibody in unprimed subjects. On the other hand, both whole-virus and disrupted-virus vaccines were effective in inducing antibody responses in immunologically primed subjects. Modern vaccines prepared by suitable methods contain little nonviral protein and endotoxins. Adverse reactions were more closely associated with the content of intact virus particles.

Influenza virus vaccine had been produced in embryonated hens' eggs for many years before avian leukemia viruses, avian adenoviruses, and mycoplasma were known to be frequent contaminants of eggs. Even after the discovery of these contaminants, however, tests for their presence in inactivated virus suspensions have been consistently negative, indicating that the organisms have been absent or killed by the inactivation processes generally used. The present requirements, therefore, do not demand the use of eggs known to be free from avian leukemia viruses, avian adenoviruses, and mycoplasma, or the testing of vaccine for the presence of these organisms. It is important, however, that any new inactivation process used in influenza vaccine production should be shown to kill such organisms.

The present requirements are applicable to preparations consisting of whole or disrupted virus, grown in fertile eggs and intended to be given parenterally. If other tissue sources were to be used for vaccine virus cultivation, these requirements would need modification.

A number of adjuvants have been used as a means of enhancing the immune response to inactivated influenza vaccine. Until further
evidence of the safety and potency of adjuvant vaccines is available, requirements for their preparation and control cannot be formulated.

In emergencies, e.g., when an influenza epidemic is expected, the abnormal demand for vaccine may create difficulties in fulfilling these requirements. No attempt has been made to indicate in what respect these requirements may be modified, since under these circumstances each national health authority must exercise its own judgement.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in type of normal size have been written in the form of requirements, so that, if a health administration so desires, these parts as they appear may be used as definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance. In order to facilitate the international distribution of vaccine made in accordance with these requirements, a summary protocol for the recording of the results of the tests is included (see pages 165-170).

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning inactivated influenza vaccine, it is recommended that a clause should be included permitting modifications of the manufacturing requirements on condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements formulated below. The World Health Organization should then be informed of the action taken.

The terms “national control authority” and “national control laboratory”, as used in these requirements, always refer to the country in which the vaccine is manufactured.

PART A. MANUFACTURING REQUIREMENTS

1. DEFINITION

1.1 International name and proper name

The international name shall be “Vaccinum Influenzae Inactivatum”. The proper name shall be the equivalent of the international name in the language of the country of origin.
The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 Descriptive definition

Vaccinium Influenzae Inactivatum shall consist of a suspension of the antigens of influenza virus types A and B, either individually or combined, grown in suitable embryonated hens' eggs and inactivated by a suitable method. The vaccines may contain whole virus or antigens obtained from the virus particles by partial or complete disruption by chemical means. Some vaccines may contain the purified surface antigens of the virus (haemagglutinin and neuraminidase). The preparation shall satisfy all the requirements formulated below. In some countries mineral carriers such as aluminium hydroxide, phosphate, or oxide or calcium phosphate have been added to the vaccine.

1.3 Reference materials for haemagglutinin

WHO influenza reference haemagglutinin antigens are prepared and distributed by the National Institute for Biological Standards and Control, London. They are preparations antigenically representative of the virus strains that are likely to be included in current vaccines. They are distributed on demand when a new influenza virus appears and the likelihood of its spread throughout the world makes desirable its inclusion in a vaccine.

The preparations contain a calibrated quantity of haemagglutinin of influenza virus measured in micrograms of haemagglutinin antigen per millilitre. The calibrations are performed at the National Institute for Biological Standards and Control, London, by single radial diffusion tests (3) with purified virus of known haemagglutinin antigen concentration. The reference preparations are used to calibrate the haemagglutinin content of national reference preparations for use in the manufacture and laboratory control of inactivated influenza vaccines by an in vitro immunodiffusion test.

These preparations, together with the specific antihaemagglutinin sera, may be obtained from the National Institute for Biological Standards and Control for the purposes of such tests.

1.4 Terminology

Seed lot. A quantity of virus processed together, fully characterized, and of uniform composition. In each manufacturing
establishment a primary seed lot is that from which secondary seed lots are prepared. Vaccine is not more than two passages removed from the secondary seed lot.

*Monovalent virus pool.* A quantity of virus suspension derived from eggs that were inoculated with the same virus strain, incubated and harvested together or in successive sessions.

*Monovalent bulk.* A quantity of inactivated, concentrated, and purified material derived from one or more monovalent virus pools that have been processed under similar conditions.

*Final bulk.* The finished vaccine prepared from one or more monovalent bulk vaccines present in the container from which the final containers are filled. It may contain one or more virus strains.

*Filling lot.* A collection of sealed final containers that have been filled in one working session from a single final bulk. They are uniform with respect to the risk of contamination during filling and sealing.

2. GENERAL MANUFACTURING REQUIREMENTS

The general requirements for manufacturing establishments contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 11) shall apply to establishments manufacturing inactivated influenza vaccine, with the addition of the following.

The areas where processing of inactivated influenza vaccine takes place shall be separate from those where work with live influenza virus is performed.

3. PRODUCTION CONTROL

3.1 Control of source materials

Strains of influenza virus used in the production of inactivated influenza vaccine shall be identified by historical records, which shall include information on the origin of the strains and their subsequent manipulation. The virus strain shall not have been passaged in continuous cell lines. If virus strains isolated in eggs are not available it may be necessary to use an isolate from suitable cell cultures. In this case the virus strain should be passaged in eggs using multiple
terminal dilution passages. The cell cultures permitted and the number of passages should be approved by the national control authority. Only strains that have been approved by the national control authority shall be used. It is now common practice to use recombinant strains giving high yields of the appropriate surface antigens. Where such strains are used, the parent high yield strain and the method of preparing the recombinant should be approved by the national control authority.

Suitable strains including recombinant strains for vaccine production are available from: Biologicals, World Health Organization, 1211 Geneva 27, Switzerland. Only recombinant strains, originating from an experienced, specialized laboratory should be used.

3.1.1 Identity of virus strain

Each manufacturer shall identify the haemagglutinin and neuraminidase antigens of the vaccine virus strains by suitable serological tests.

3.1.2 Virus propagation

Influenza virus used in the production of inactivated influenza vaccine shall be propagated in embryonated hens’ eggs. The eggs shall be derived from healthy flocks.

3.1.3 Seed lot system

The production of vaccine shall be based on the seed lot system. Each seed lot shall be identified as influenza virus of the appropriate strain by methods acceptable to the national control authority.

3.1.4 Tests on seed lots

The seed virus shall be shown to be free from demonstrable adventitious viable microorganisms by appropriate tests according to the requirements of Part A, sections 5.2 and 5.3, of the revised Requirements for Biological Substances and other Sets of Recommendations No. 6 (General Requirements for the Sterility of Biological Substances) (3, pp. 49–52).

The seed virus should be prepared in specific-pathogen-free eggs and tested for the presence of avian leukosis virus and
adenoviruses. The seed lot should be stored at a temperature lower than \(-60\)°C unless it is in the lyophilized form, in which case it should be stored at a temperature lower than \(-20\)°C.

3.2 Production precautions

The general production precautions formulated in Part A.A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 15) shall apply to the manufacture of inactivated influenza vaccine, with the addition of the following.

Only allantoic and amniotic fluids shall be harvested.
Penicillin or streptomycin shall not be used at any stage of manufacture of the vaccine.

Minimal concentrations of other suitable antibiotics may be used. It should be kept in mind, however, that cross-allergenic reactions sometimes occur between cephalosporin, neomycin, and the penicillins.

In the case of vaccines that involve the splitting of the virus by chemical means, the splitting and the concentration of chemicals shall be carried out by methods approved by the national control authority.

3.3 Inactivation of monovalent virus pools

3.3.1 Time of inactivation

Inactivation of monovalent virus pools shall be initiated as soon as possible after their preparation.

However, if storage before inactivation is unavoidable, they should be held at a temperature of \(5\)°C±\(3\)°C for not longer than 5 days.
Before inactivation of monovalent virus pools, samples should be taken and tested for bacterial and fungal contamination. Such tests serve to indicate the reliability of the source of eggs.

3.3.2 Inactivation procedure

The virus in the monovalent virus pools shall be inactivated by a method that has been demonstrated to be consistently effective in the hands of the manufacturer and has been approved by the national control authority. The inactivation process shall also have been
shown, to the satisfaction of the national control authority, to be capable of inactivating avian leukemia viruses and mycoplasma. If the virus pool is stored after inactivation, it shall be held at a temperature of 5°C ± 3°C. The inactivation procedure should cause minimum alteration of the haemagglutinin and neuraminidase.

If formalin (40% formaldehyde) or beta-propiolactone is used, the concentration should not exceed 1:2000 at any time during inactivation.

3.4 Concentration and purification procedures

The monovalent material shall be concentrated and purified by high-speed centrifugation or other suitable method approved by the national control authority, either before or after the inactivation procedure.

The aim is to separate the virus from other constituents in the allantoic fluids with the maximum efficiency. It is advisable to concentrate and purify the virus under optimum conditions to preserve its antigenic properties.

In some countries, electron microscopy is used to check the purity of the virus suspension. Another test for purity consists in injecting vaccine into mice and testing their sera for antibodies against egg protein.

3.5 Control of monovalent bulk

3.5.1 Test for effective inactivation

The monovalent bulk shall be tested for the presence of viable influenza virus by inoculation of embryonated hens’ eggs, with a method approved by the national control authority.

A suitable method consists in inoculating 0.2 ml of undiluted monovalent bulk and 1:10 and 1:100 dilutions of the monovalent bulk into the allantoic cavities of fertile eggs (a group of 10 eggs in each case), and incubating at 33-37°C for 3 days. At least 8 of the 10 eggs should survive at each dosage level. Harvest 0.5 ml of the allantoic fluid from each surviving egg, pooling the fluid harvested from each group. Then 0.2 ml of the pooled fluid is inoculated, undiluted, into a further 3 groups each of 10 fertile eggs. Haemagglutinin activity should not be detected in these new groups of eggs.

In some countries, a survival proportion as high as 90% during incubation may not be achievable. The national control authority should decide in this case about the limits of acceptability.
3.5.2 Tests for the concentration of haemagglutinin antigen

The content of haemagglutinin in the monovalent bulk shall be determined by an immunodiffusion technique, such as single-radial-immunodiffusion or immunolectrophoresis. The test shall be made in comparison with the WHO influenza reference haemagglutinin antigen or with a national preparation calibrated against it.

For certain types of vaccine, immunodiffusion techniques may not give reproducible results. In such cases, other known methods may be used.

3.5.3 Tests for the presence of neuraminidase

In some countries, a test is included for the presence of neuraminidase. The ratio of haemagglutinin to neuraminidase should be similar to that of the expected values for the virus strain and method of vaccine production used, neuraminidases of different strains varying markedly in their stability during processing.

3.6 Control of final bulk

3.6.1 Preservatives and other substances added

In preparing the final bulk, only preservatives or other substances, including diluents, approved by the national control authority shall be added. Such substances shall have been shown by appropriate tests not to impair the safety or effectiveness of the product in the concentrations used, and they shall not be added before samples have been taken for any tests that would be affected by the substances added.

If mercury-containing preservatives are used, the final bulk should be tested for mercury content.

3.6.2 Test for influenza virus

The final bulk shall be tested for the presence of viable virus by an approved method (see Part A, section 3.5.1).

3.6.3 Test for content of haemagglutinin antigen

The haemagglutinin concentration in the final bulk shall be determined as described in Part A, section 3.5.2, unless such a test is done on each filling lot.
In some countries, the concentrations of haemagglutinin contained in the vaccine, particularly with certain virus strains, are such that this test is difficult to include at this stage. It may be omitted on the advice of the national control authority and, in such cases, the measurement of haemagglutinin content is based on the test on the monovalent bulk (see Part A, section 3.5.2).

3.6.4 Test for identity of the virus antigens

The identity of the haemagglutinin antigen or of the haemagglutinins in the vaccine shall be determined by an immunological technique, such as immunodiffusion or haemagglutination inhibition, using the appropriate specific immune serum. For adsorbed vaccines, the presence of specific antibodies in sera from immunized animals may be used.

In some countries, a test to identify the specific neuraminidase antigen is also included.

3.6.5 Sterility tests

Each final bulk shall be tested for sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances and other sets of Recommendations No. 6 (General Requirements for the Sterility of Biological Substances) (3, page 48).

3.6.6 Protein nitrogen content

The protein nitrogen content shall be determined, and the upper limit shall be no greater than that approved by the national control authority (see Part B, section 1).

The protein nitrogen content of the final bulk should be as low as possible in relation to the haemagglutinin content of the vaccine. Values of less than 25 µg of protein nitrogen per virus strain per single human dose are obtainable.

For the final bulk, the upper limit should be less than 50 µg of protein nitrogen per single human dose.

3.6.7 Test for endotoxin

In some countries, tests for endotoxins—the Limulus amoebocyte lysate test, the rabbit test, or the leukopenia test in
mice—are included. The endotoxin content is measured against a reference preparation, the permissible level of the endotoxin being determined by the national control authority.

3.6.8 Tests for chemicals used in production

The concentration of each detergent, organic solvent, inactivating agent, and preservative remaining in the final vaccine shall be determined by methods approved by the national control authority. These concentrations shall not exceed upper limits specified by the national control authority. For preservatives, the concentration shall not be less than a lower limit specified by the national control authority.

Tests for chemicals that are not used during preparation of the final bulk may also be performed on monovalent bulks.

4. FILLING AND CONTAINERS

The requirements concerning filling and containers given in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 16) shall apply.

Single and multiple-dose containers may be used. If multiple-dose containers are used, a suitable preservative, approved by the national control authority, should be incorporated.

5. CONTROL TESTS ON FINAL PRODUCT

5.1 Identity test

An identity test shall be performed on at least one labelled container from each filling lot by a method approved by the national control authority.

5.2 Sterility tests

Each filling lot shall be tested for sterility according to the requirements given in Part A, section 5.2, of the revised Requirements for Biological Substances and other Sets of Recommendations No. 6 (General Requirements for the Sterility of Biological Substances) (3, page 49).
5.3 Test for content of virus antigen

A test shall be performed on each filling lot according to the requirements given in Part A, section 3.5.2, unless such a test has been performed on the final bulk (see Part A, section 3.6.3).

5.4. Inocuity tests

Each filling lot shall be tested for abnormal toxicity by appropriate tests by means of parenteral injections in mice and guinea-pigs. The test procedures shall be those approved by the national control authority.

5.5 Inspection of final containers

Each container in each filling lot shall be inspected, and those showing abnormalities shall be discarded.

6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 17) shall apply.

7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 18) shall apply.

8. LABELLING

The requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 18) shall apply, with the addition of the following.

If the product is issued in a multiple-dose container, the label on the container shall state the number of doses.
The label on the carton enclosing one or more final containers or the leaflet accompanying the container shall include the following additional information:

- A statement that the virus was propagated in hens' eggs;
- the method used for inactivating the virus;
- the name and maximum quantity of any antibiotic present in the vaccine;
- the strains included in the vaccine (for the nomenclature to be used for designating strains, see 7);
- the quantity of haemagglutinin per dose for each strain expressed as micrograms of haemagglutinin per dose; and
- the name and concentration of the preservative, if added.

9. DISTRIBUTION AND TRANSPORT

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and control Laboratories) (6, page 18) shall apply.

10. STORAGE AND EXPIRY DATE

The requirements given in Part A, section 10, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 19) shall apply.

10.1 Storage conditions

Inactivated influenza vaccine shall be stored at a temperature of 5°C±3°C.

10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority.

The expiry date should be not more than two years after the last satisfactory test for virus antigen content (see Part A, sections 3.6.3 and 5.3), provided that the vaccine has been stored continuously at a temperature of 5°C±3°C. It is desirable that the expiry date should not exceed one year from the date of issue by the manufacturer.
PART B. NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories, contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 19), shall apply.

The national control authority shall give directions to manufacturers concerning the influenza virus strains to be used, the virus antigen content (haemagglutinin), and the recommended human dose.

2. RELEASE AND CERTIFICATION

A vaccine lot shall be released only if it fulfils Part A of these requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishments and shall certify that the lot of vaccine in question meets all national requirements as well as Part A of these requirements. Furthermore, this certificate shall state the date of the last satisfactory determination of content of virus antigen, the lot number, the number under which the lot was released, and the number appearing on the labels of containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of inactivated influenza vaccine between countries.

3. CLINICAL EVALUATION OF INFLUENZA VACCINES

In the case of a new manufacturer or of a significant change in the manufacturing process, the national control authority shall ascertain that the vaccine is safe and effective by arranging for studies in man of some of the lots of vaccine that have satisfied the above-mentioned requirements. Such studies shall include the assessment of the immune responses and reactions in various age groups. Studies to demonstrate the safety and efficacy of the vaccine shall be carried out and evaluated before approval for release of any lots of the vaccine for general use.
Primary/Secondary Seed Lot Type A

Name and address of manufacturer

Laboratory reference No. of lot

Date when the processing was completed

Information on manufacture

1. Virus used to inoculate eggs for the manufacture of the lot
   (a) strain and substrain
   (b) passage level
   (c) source and reference No.
   (d) remarks

2. Results of sterility tests

3. Conditions of storage

Primary/Secondary Seed Lot Type B

Name and address of manufacturer

Laboratory reference No. of lot

Date when the processing was completed

Information on manufacture

1. Virus used to inoculate eggs for the manufacture of the lot
   (a) strain and substrain
   (b) passage level
   (c) source and reference No.
   (d) remarks

2. Results of sterility tests
3. Conditions of storage

**Monovalent Virus Pool Type A**

Name and address of manufacturer
Laboratory reference number of virus pool

1. Virus used to inoculate eggs*
   (a) primary seed strain and source
   (b) passage level of primary seed
   (c) secondary seed lot, reference No., and source

2. Date of inoculation
3. Date of harvesting allantoic fluids
4. Conditions of storage before inactivation
5. Date of inactivation
6. Method of inactivation

7. Concentration of inactivating agent
8. Conditions of storage after inactivation
9. Concentration/purification procedure

10. Antibiotics used during preparation, if any

**Tests on monovalent bulk**

1. Test for absence of viable influenza virus
   No. of eggs inoculated

* If there are more than four virus pools in the monovalent bulk, the relevant data should be given on a separate sheet.
Inactivation time and temperature

Results

2. Test for presence of neuraminidase (if performed)

Method

Results

**Monovalent Virus Pool Type B**

Name and address of manufacturer

Laboratory reference number of virus pool

1. Virus used to inoculate eggs
   - (a) primary seed strain and source
   - (b) passage level of primary seed
   - (c) secondary seed lot, reference No., and source

2. Date of inoculation

3. Date of harvesting allantoic fluids

4. Conditions of storage before inactivation

5. Date of inactivation

6. Method of inactivation

7. Concentration of inactivating agent

8. Conditions of storage after inactivation

9. Concentration/purification procedure

10. Antibiotics used during preparation, if any
Tests on monovalent bulk

1. Test for absence of viable influenza virus
   No. of eggs inoculated
   Incubation time and temperature
   Results

2. Test for presence of neuraminidase
   (if performed)
   Method
   Results

Final Bulk

1. Name and address of manufacturer
2. Identification of final bulk
3. Identification of monovalent bulk vaccines used to prepare final bulk
4. Date of manufacture

Control of final bulk

1. Preservative(s) added and concentration
2. Any other substances added and concentration
3. Test for viable virus
   No. of eggs inoculated
   Dilution and dose per egg
   Incubation time and temperature
   Results
4. Test for content of haemagglutinin antigen
   Method
   Results

5. Test for identity of virus antigens
   Method
   Results
   (Result of test for specific neuraminidase, if performed)

6. Test for sterility
   Results

7. Protein nitrogen content
   ug per single human dose

8. Test for endotoxin
   Method
   Results

9. Tests for chemicals used
   Results

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Final Product

1. Identity tests
   Method
   Results
2. Sterility tests
   Results

3. Test for virus antigen content (unless done on final bulk)
   Method
   Results

4. Innocuity tests
   No. and species of animals
   Doses injected
   Period of observation
   Results

5. Inspection of final container
   Results

6. Other tests

7. Remarks

Signature of head of laboratory

Certification by person taking overall responsibility for production of the vaccine:
I certify that lot No. ______ of vaccine satisfies Part A of the WHO Requirements for Influenza Vaccine (Inactivated).

Signature

Name typed

The protocol must be accompanied by a sample of the label and a copy of the leaflet.
REQUIREMENTS FOR INFLUENZA VACCINE (LIVE)
(Requirements for Biological Substances No. 28)

INTRODUCTION

The possibility of controlling epidemic influenza by the use of live attenuated vaccine given by the intranasal route has been investigated. For some years, the principle has had a sound basis, having been tested in infectious diseases, notably poliomyelitis, in which an attenuated virus given by the natural route of infection has been shown to give protection without disease. By analogy, it should be possible to initiate a benign infection by influenza virus with an attenuated strain that would give protection against the prevalent infectious wild strain. This approach has been tried on a number of occasions with mixed success. Some trials showed great promise, whereas others gave results indicating potential dangers that might arise from the use of these products because of insufficient knowledge of the fundamental principles involved.

The aim is to produce from a wild strain an attenuated virus that will be stable and not cause influenza when given to susceptible individuals on a wide scale. Thanks to modern technology, there are now tests that can answer many of the questions unresolved for decades.

An attenuated influenza vaccine made from a live attenuated strain of virus is now used in so many countries that it is appropriate to formulate WHO Requirements for such a vaccine.

The international requirements for influenza vaccine (live) have been fitted into the framework adopted in the Requirements for Biological Substances No. 1-27, already published by WHO (4, page 142). A group of WHO consultants and staff members (listed on page 192) drafted them, taking into account the regulations and requirements for the manufacture and control of influenza vaccine (live) in a number of countries, as well as information from published and unpublished reports. In addition, opinions and data have been received from a number of experts, whose assistance is acknowledged (see page 192).

GENERAL CONSIDERATIONS

There are many potential problems related to the development of a safe and effective live influenza vaccine. It is important, therefore,
to characterize any candidate vaccine in carefully designed laboratory and clinical studies before its widespread use is considered. The studies should be designed so as to confirm that the vaccine virus is attenuated, is not transmitted to susceptible contacts, is genetically stable, is immunogenic, and has suitable laboratory markers of attenuation. In addition, the appropriate nature of the vaccine strain will need to be reviewed continuously to ensure the development of vaccines that contain the currently prevalent antigens and have been demonstrated to be safe and effective. Therefore, the development and control of live influenza vaccines require a long-term commitment to laboratory and clinical studies, and it is recommended that only countries with adequate resources for these studies should consider the manufacture and control of these products.

The development of a suitable vaccine comprises a number of essential stages:

1. Primary investigations into the attenuation are carried out in the laboratory on animals (ferrets). The results, compared with those of inoculating wild virus, make it possible to decide whether limited trials in human beings are acceptable. There are also a number of tests in vitro (genotypical characterization by RNA-RNA hybridization, culture of tracheal epithelium) that may provide indications on attenuation. The results of these tests should confirm those of animal tests.

2. Limited trials in human beings, performed in an environment offering the maximum guarantee of isolation and discipline. These trials are to make sure that the virus is not excreted at too high a titre and too long, that the virus is not transmitted, that the reactions are acceptable, and that a sufficient antibody level is achieved.

3. Human trials on a larger scale, with subjects in good health and of different ages, if the previous trials were satisfactory. These trials serve to specify the necessary dose and to prove the innocuity of the vaccine for different age groups and the absence of transmission. A placebo group is essential to evaluate the incidence and intensity of reactions. Pulmonary function should be evaluated in vaccinated subjects.

4. Innocuity tests in the population at high risk, for which the vaccine is specially intended (subjects with chronic bronchitis and asthma).

In developing a new vaccine strain, methods of attenuation that have been shown to produce safe and immunogenic vaccine should
be used. Over the last few years several new methods of attenuation have been developed, involving host-range infectivity, temperature sensitivity, or cold-adapted mutant strains. Candidate viruses may be derived by the selection from wild virus populations of mutants possessing suitable characteristics or by the genetic transfer of the characteristics from standard parent attenuated strains to wild strains by recombination (gene reassortment). Techniques of gene analysis of candidate viruses should be employed for their selection and laboratory characterization. Genetic stability is an extremely important characteristic for the selection of vaccine strains and should be determined in order to ensure that reversion to virulence does not occur during passage in man.

Recombination between attenuated parent strains and the new wild virus followed by careful selection has been shown to be an effective and rapid method of producing attenuated strains with the surface antigens of the wild parent. Vaccine strains should be adequately cloned by multiple terminal dilution passages in specific pathogen-free eggs or by other acceptable methods.

The availability of reliable markers of attenuation is important. It may be desirable to select vaccine strains that, in addition to markers correlating with human virulence, also possess other stable markers that do not co-vary with virulence for man and, therefore, might be used to identify vaccine strains in epidemiological surveillance.

Wide-scale use of live influenza vaccines in large field trials or in routine immunization has demonstrated that satisfactory products can be obtained. However, mild upper respiratory reactions in some persons and occasional benign systemic reactions have been observed. The vaccines are usually given intranasally and the virus replicates in the nasopharyngeal mucosa. The virus is shed by some vaccinated persons, but excretion is usually less in quantity and shorter in duration than is the case during infections caused by virulent influenza viruses. The possibility of virus transmission to susceptible contacts, therefore, is much smaller than it is for wild virus.

Influenza virus is unique from the viewpoint of antigenic change, although live vaccines appear to confer a degree of cross-protection against related strains within a common haemagglutinin. The use of a vaccine strain antigenically identical to the naturally prevalent virus strain is expected to provide optimum protective efficacy. Since the interval between the appearance of a new influenza virus variant and its spread throughout the world may be a matter of months, the development of an appropriate vaccine strain must be rapid.
The testing of the lots of experimental vaccine that may be established as the seed from which vaccine is prepared should take into account the limited period in which a new strain has to be developed. Therefore, some tests included for routine production lots may have to be amended for these experimental vaccine lots. The subsequent testing in man should also be designed to allow the most informative evaluation of the safety and immunogenicity of the strain in the shortest possible time. Vaccine strains should be evaluated in clinical studies including representative population groups for which vaccine may be recommended, e.g., children and high-risk adults.

Where appropriate, the technology and experience acquired over the last two decades in the production and control of all live virus vaccines should be applied to live influenza vaccines. As for all live vaccines, the substrate on which the virus is propagated is important. Since influenza virus is usually produced in embryonated hens' eggs, the eggs should be obtained from closed flocks that are continuously monitored for known pathogenic agents. These flocks are now available in many countries, and the eggs — or cell cultures derived from them — have been widely used in the production of measles vaccine and other vaccines produced in avian substrates. Furthermore, stability after lyophilization is comparable to that of other live virus vaccines.

In addition to the many technical problems associated with the rapid development of suitable attenuated vaccine strains, consideration should be given also to the need for special administrative arrangements for licensing, which involve the submission of data and their review by national control authorities. The usual periods of time required for licensing procedures will need to be shortened considerably if appropriate live vaccines are to be made available when needed soon after the emergence of a new variant. Close collaboration between manufacturers and the national control authorities is required, particularly during the phases of development, production, and testing of the initial batches of live vaccines.

In emergencies, such as an influenza pandemic, the abnormal demands for vaccine may create difficulties if all the tests in these requirements are to be carried out. No attempt has been made to indicate in what respect these requirements may be modified, since such decisions are the responsibility of national control authorities.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in type of normal size have been written in the form of requirements so that, if a health administration
so desires, these parts as they appear may be used as definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance. In order to facilitate the international distribution of vaccine made in accordance with these requirements, a summary protocol for the recording of the results of the tests is included (see pages 187-192).

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning live influenza vaccine, it is recommended that a clause should be included permitting modifications of the manufacturing requirements on condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements formulated below. The World Health Organization should then be informed of the action taken.

The terms “national control authority” and “national control laboratory”, as used in these requirements, always refer to the country in which the vaccine is manufactured.

PART A. MANUFACTURING REQUIREMENTS

1. DEFINITION

1.1 International name and proper name

The international name shall be “Vaccinum Influenzae Vivum”. The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 Descriptive definition

Vaccinum Influenzae Vivum is a preparation of live attenuated influenza virus grown in suitable embryonated hens’ eggs.

1.3 International standards and international reference preparations

Since no international reference preparations of live attenuated influenza virus are available, no requirements based
on comparisons with such preparations can be formulated at present.

National control authorities should provide a reference preparation of live attenuated influenza virus for use in tests of virus infectivity (see Part A, sections 3.5.3 and 5.3).

1.4 Terminology

*Vaccine strain.* An attenuated influenza virus that has been shown to be immunogenic and safe during clinical trials in human beings.

*Seed lot.* A quantity of virus derived from the vaccine strain processed together, fully characterized, and of uniform composition. Seed lots are not more than two passages removed from the original vaccine strain.

*Single harvest.* A quantity of virus suspension harvested from eggs from the same source that were inoculated with the same virus strain and incubated and harvested together or in successive batches.

*Bulk suspension.* Material prepared from one or more single harvests and before filling into final containers.

*Final bulk.* The finished preparation present in the container from which the final containers are filled. The final bulk may be prepared from one clarified bulk suspension, or from a blend of clarified bulk suspensions, or from a dilution thereof.

*Filling lot (final lot).* A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling and drying. A filling lot must, therefore, have been filled in one working session and have been dried together.

*Egg infectivity dose 50% (EID<sub>50</sub>).* The quantity of a virus suspension that will infect 50% of embryonated eggs.

2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (§, pp. 11–22) shall apply to establishments manufacturing live influenza vaccine, with the addition of the following.

Production areas shall be decontaminated before they are used for the manufacture of live influenza vaccine.

The production of live influenza vaccine shall be conducted by staff who have not handled other infectious microorganisms or
animals on the same working day. The staff shall consist of persons who shall have been examined medically and found to be healthy. Staff with respiratory infections shall be excluded.

Only eggs obtained from specific pathogen-free flocks approved by the national control authority for the production of live influenza vaccine shall be introduced into or handled in the production area.

Visitors and persons not directly concerned with the production process shall not be permitted to enter the production area.

Particular attention shall be paid to the recommendations given in Part A, section 1, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 13) regarding the training and experience of the persons in charge of production and testing and of those assigned to various areas of responsibility in the manufacturing establishment, as well as to the registration of such personnel with the national control authority.

3. PRODUCTION CONTROL

The general production precautions formulated in the requirements of Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 15) shall apply to the manufacture of live influenza vaccine.

3.1 Control of source materials

3.1.1 Virus strains

The strain of influenza virus used in the production of live influenza vaccine shall be identified by historical records, which shall include information on the origin of the strain and on the method used to attenuate it. The strain shall be shown to be immunogenic and safe, and only strains that are approved by the national control authority shall be used.

3.1.2 Substrate for virus propagation

Influenza virus used in the production of live influenza vaccine shall be propagated in specific pathogen-free eggs approved by the
national control authority. All information on the flock used as the source of eggs shall be available to the national control authority.

The eggs shall be derived from closed flocks continuously monitored for the absence of avian encephalomyelitis, avian adenovirus, avian reticulooendotheliosis virus, Newcastle disease, fowlpox, avian infectious bronchitis, infectious bursal agent, laryn-gotracheitis, influenza type A infection, leukosis virus, Marek disease herpetovirus, avian reovirus, Mycoplasma species—e.g., *M. gallisepticum* and *M. synoviae*, *Salmonella* species—e.g., *S. pullorum*, EDS67, and *Mycobacterium tuberculosis* (of avian origin). The flock shall be free from these infectious agents.

3.1.3 Seed lot system

The production of vaccine shall be based on a seed lot system.

Each seed lot shall be identified as containing the appropriate virus. If not lyophilized, the seed lot shall be stored at a temperature lower than $-60^\circ$C; if lyophilized, the storage temperature shall be lower than $-20^\circ$C.

All single harvests shall contain influenza virus derived only from eggs inoculated with the same seed lot. The virus in the final vaccine shall be not more than three passages removed from the vaccine strain used in the preparation of a vaccine shown to be immunogenic and safe in man.

3.1.4 Tests on seed lots

The seed lot used for the production of vaccine shall be free from extraneous agents. The eggs used shall conform to the tests specified in Part A, section 3.2. The seed lot shall be produced on the substrate specified in Part A, section 3.1.2, and shall be controlled by means of the tests mentioned in Part A, section 3.4.

If the vaccine strain has been passaged in substrates other than specific pathogen-free eggs, the national control authority should specify the additional tests for the detection of the adventitious agents that may have been present in the substrate used.

3.2 Tests on control eggs

Of each batch of eggs used for propagation of the virus 2% (or at least 20) shall be held as uninoculated controls and incubated for the
same time and at the same temperature as the inoculated eggs. At the time of harvesting the virus from the inoculated eggs, allantoic fluids shall be taken from the uninoculated eggs and examined for haemagglutinating agents (see Part A, section 3.2.1).

To ensure freedom from other avian infectious agents, the greatest reliance is placed on the continuous monitoring of the flock from which the eggs are obtained. However, national control authorities should include additional tests for microorganisms if necessary. Tests for avian leukosis virus and inoculation of the control fluids on choriointoallantoic membranes are essential.

3.2.1 Tests for haemagglutinating agents

A sample of 0.25 ml of allantoic fluid taken from each egg shall be tested by the addition of chick erythrocytes for haemagglutinating agents both directly and after one passage through specific pathogen-free eggs. The details of the test shall be approved by the national control authority.

3.3 Harvesting

Only the allantoic fluids from the inoculated eggs shall be harvested. No penicillin or streptomycin shall be present in the harvested fluids or added at the time of harvesting or subsequently.

If antibiotics are used, it should be kept in mind that allergic cross-reactions sometimes occur between cephalosporin, neomycin, and the penicillins.

It is a wise precaution to pool the allantoic fluids from a limited number of eggs (e.g., 30–50) and to test these small pools for sterility and virus titre before blending into a single harvest. Only small pools that are free from bacteria and fungi should be blended into the harvest.

The pools should be stored at a temperature of 5°C–5°C. Small quantities of other antibiotics may be added, with the approval of the national control authority. However, if antibiotics are added, samples for sterility testing should be taken before the antibiotic is added, and all small pools shown to be contaminated should be discarded.

3.4 Tests on single harvest

At the time of pooling the allantoic fluids and before clarification, samples shall be set aside for examination for extraneous agents. If
the samples are not tested immediately, they shall be stored at 
−60°C or below.

For the purposes of the tests required on neutralized virus 
harvests, the hyperimmune serum shall be of non-avian, non-simian, 
non-human origin. The virus used for the production of the hyper-
immune serum shall be grown either in non-avian cell cultures or in 
specific-pathogen-free eggs. If eggs are used, they shall be obtained 
from a flock different from that used to supply the production 
eggs.

The virus pool passes the test if there is no evidence of the 
presence of any adventitious agents attributable to the virus pool 
when tested according to the requirements of this section and its 
subsections.

3.4.1 Tests in animals

After neutralization of the influenza virus by the hyperimmune 
serum, the virus pool sample shall be inoculated into animals as 
described in sections 3.4.1.1 and 3.4.1.2 below.

3.4.1.1 Tests in adult mice. A total volume of at least 10 ml of the 
virus pool shall be tested by inoculating 0.5 ml of the virus-serum 
mixture into each of 20 or more adult mice by the intraperitoneal 
route. A total volume of at least 0.6 ml of the virus shall be tested in 
20 or more mice by inoculating 0.03 ml into each mouse by the intra-
cerebral route. The same mice may be used for both routes of inocu-
lation.

The mice shall be observed for at least 21 days. All mice that die 
after the first 24 h of the test, or that have been sacrificed after 
showing signs of illness, shall be autopsied and examined for 
evidence of viral infection, by macroscopic observation and by 
subinoculation of appropriate tissue suspensions by the intracerebral 
and intraperitoneal routes into at least 5 additional mice, which shall 
be observed for 21 days.

The virus pool shall be considered to have passed the test if at 
least 80% of the original inoculated mice survive the observation 
period and if no mice show evidence of infection with adventitious 
transmissible agents attributable to the virus pool.

3.4.1.2 Tests in suckling mice. A total volume of at least 2 ml of 
virus shall be tested by inoculating each of 20 or more suckling mice
by the intraperitoneal route with 0.1 ml of the virus/serum mixtures. A total volume of at least 0.1 ml of virus shall be tested in 10 or more newborn mice by inoculating 0.01 ml into each mouse by the intracerebral route.

The mice shall be observed daily for at least 21 days. All mice that die after the first 24 hrs. of the test or that have been sacrificed after showing signs of illness shall be autopsied and examined to determine the cause of death or illness.

In some countries a subinoculation is appropriate, and in others an additional blind passage is made of a suspension of the pooled emulsified tissue (minus skin and visera) of all mice surviving the original 21-day test.

The virus pool shall be considered to have passed the test if at least 80% of the original inoculated mice survive the observation period and if no mice show evidence of infection with adventitious transmissible agents attributable to the virus pool.

3.4.2 Tests in cell cultures

After neutralization of the influenza virus by the hyperimmune serum, the virus pool sample shall be tested in cell cultures as described below (sections 3.4.2.1–3.4.2.3).

3.4.2.1 Tests in human cells. A sample of at least 5 ml of the virus pool after neutralization shall be inoculated on cell cultures of human origin. The cell cultures and the period of observation shall be approved by the national control authority. None of the cell cultures shall show evidence of the presence of any adventitious agent.

3.4.2.2 Tests in simian cells. A sample of at least 5 ml of the virus pool after neutralization shall be inoculated on simian cell cultures. The cell cultures and the period of observation shall be approved by the national control authority. None of the cell cultures shall show evidence of the presence of any adventitious agent.

3.4.2.3 Test in chicken cells. A sample of at least 5 ml of the virus pool after neutralization shall be inoculated on chicken cell cultures. The cell cultures and the period of observation shall be approved by the national control authority. None of the cell cultures shall show evidence of the presence of any adventitious agent. The absence of
avian leukemia viruses shall be ascertained by testing. The chicken cell cultures may be used also for this purpose.

3.4.3 Tests in embryonated eggs

A sample of at least 10 ml of each single harvest after neutralization shall be tested in a group of embryonated hens' eggs by the allantoic route of inoculation, and a similar sample in a separate group of eggs by the yolk sac route of inoculation, using at least 0.25 ml of the virus/serum mixture per egg for each route of inoculation. The incubation of the eggs and the observation time shall be approved by the national control authority.

The virus pool passes the test if there is no evidence of the presence of any adventitious agents attributable to the virus pool.

3.4.4 Tests for bacteria, fungi, and mycoplasma

The single harvest shall be tested for the presence of bacteria, fungi, and mycoplasmas according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances and other sets of Recommendations (General Requirements for the Sterility of Biological Substances) (3, page 48).

3.4.4.1 Test for mycobacteria. Each single harvest shall be tested for the presence of mycobacteria by culture methods appropriate for the detection of the organisms most likely to be found in the eggs used.

It is common practice to concentrate the virus harvest by centrifugation and to inoculate the pellet into guinea-pigs or on to solid media shown to be suitable for the detection of mycobacteria.

3.5 Control of bulk suspension after clarification

3.5.1 Clarification of bulk suspension

The bulk suspension shall be clarified by a method that will remove intact tissue cells.

3.5.2 Sampling

Samples of the clarified bulk suspension shall be taken immediately after clarification. If not tested immediately, the samples
shall be kept at a temperature below –60°C until the test for virus
titrations (see section 3.5.3 below) is made.

3.5.3 Virus titration

The influenza virus content in the clarified bulk suspension shall
be determined by titrations in eggs, using as a control a reference
preparation of influenza virus of known infectivity (see Part B,
section 1).

3.6 The final bulk

The operations necessary for preparing the final bulk shall be
conducted in such a manner as to avoid contamination of the
product.

The dilution and mixing procedures involved in preparing the
final bulk should be those approved by the national control
authority.

3.6.1 Added substances

Any substance that is added to the product in preparing the final
bulk shall have been shown, to the satisfaction of the national control
authority, not to impair the safety and efficacy of the vaccine in the
concentration used.

4. FILLING AND CONTAINERS

The requirements concerning filling and containers given in
Part A, section 4, of the revised Requirements for Biological
Substances No. 1 (General Requirements for Manufacturing
Establishments and Control Laboratories) (6, page 16) shall
apply.

Care should be taken to ensure that the material of which the
container is made does not adversely affect the virus content of the
vaccine under the recommended conditions of storage.

5. CONTROL TESTS ON FINAL PRODUCT

Samples shall be taken from each filling lot for the tests
mentioned in this section and its subsections.
5.1. Identity test

The virus in the final containers shall be identified by appropriate immunological methods to identify the haemagglutinin, neuraminidase, and any specific marker of attenuation of the seed virus.

The specific marker tests may also be performed on the final bulk instead of on the final product.

5.2 Tests for bacteria and fungi

Reconstituted vaccine shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5, of the Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances) (A, page 48).

5.3 Virus titration

The virus content of each of at least three ampoules selected at random from each drying lot shall be determined individually. The details of the test shall be approved by the national control authority.

This determination shall be made in terms of EID₉₀ in parallel with the determination of the virus concentration of a reference preparation of known virus titre (see Part B, section 1). The determination of the EID₉₀ per dose shall be based on at least five inoculated eggs per dilution, using tenfold dilutions.

The number of EID₉₀ per human dose of vaccine shall be determined. The requirements for virus content per human dose shall be based on clinical trials in man.

5.4 Innocuity tests

The innocuity of the product shall be tested by appropriate tests in mice and guinea-pigs by means of parenteral injections. The tests shall be those approved by the national control authority.

5.5 Residual moisture

The residual moisture in a representative sample of each freeze-dried lot shall be determined by a method approved by the national control authority.
6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 17) shall apply.

7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 18) shall apply.

8. LABELLING

The requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 18) shall apply, with the addition of the following.

The label on the container shall include the following information:

- the name(s) of the strain(s) of influenza virus (7) contained in the vaccine.

The leaflet accompanying the package shall include the following information:

- the name(s) of the strain(s) of influenza virus (7) contained in the vaccine;
- the nature and amount of any preservative or stabilizer present in the vaccine;
- the amount of virus contained in one recommended human dose for adults and children;
- the name and maximum quantity of any antibiotic present in the vaccine;
- a statement that after the vaccine has been reconstituted it should be used without delay, or, if not used immediately, stored between 2°C and 8°C for a period not exceeding 8 hrs; and
- a statement that the vaccine has been prepared in hens’ eggs.
9. DISTRIBUTION AND TRANSPORT

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 18) shall apply.

10. STORAGE AND EXPIRY DATE

The requirements given in Part A, section 10 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 19) shall apply.

10.1 Storage conditions and stability

After labelling, packaging, and distribution or issue, the vaccine shall be stored at a temperature not exceeding 8°C. The vaccines shall have been shown to maintain the virus content of the human dose for a period equal to that between the date of issue and the expiry date. The manufacturers shall have shown by accelerated degradation tests that the vaccine in the freeze-dried form is stable.

10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory test for virus concentration, the date of this test being that on which the test system was inoculated. The expiry date shall be not more than one year after the date of the last satisfactory test for virus concentration.

If experience shows that the vaccine remains stable longer, the national control authority may extend the expiry date.

PART B. NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories, contained in Part B of the revised Requirements for Biological Substances No. 1
LIVE INFLUENZA VACCINE

(General Requirements for Manufacturing Establishments and Control Laboratories) (6, page 19), shall apply.

The national control authority shall give directions to manufacturers concerning the influenza virus strains to be used for vaccine production.

The national control authority should take into consideration all information available on strains before deciding on those permitted for vaccine production.

In addition, the national control authority shall provide a reference preparation of live influenza virus to be used for checking the normal susceptibility of the titration system, and shall specify the requirements for virus content to be fulfilled in order to achieve adequate immunization of human beings with the recommended human dose.

2. RELEASE AND CERTIFICATION

A vaccine shall be released only if it fulfils Part A of the present requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify that the lot of vaccine in question meets all national requirements as well as Part A of the present requirements. The certificate shall state the date of the last satisfactory test for virus concentration, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of live influenza vaccine between countries.

SUMMARY PROTOCOL FOR INFLUENZA VACCINE (LIVE)

Based on the Requirements for Influenza Vaccine (Live)
(Requirements for Biological Substances No. 29)

Name and address of manufacturer

Lot No. of vaccine
### No. of freeze-drying lot

### Date of initiation of last test for virus concentration

### Expiry date

### Proprietary name of vaccine

### No. and volume of ampoules or vials in the lot

#### Seed virus

<table>
<thead>
<tr>
<th>Seed virus strain</th>
<th>Date when established</th>
<th>Seed lot reference No.</th>
<th>Date(s) of satisfactory test(s) for freedom from extraneous agents</th>
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</table>

#### Control of eggs

<table>
<thead>
<tr>
<th>Source of eggs</th>
<th>No. of eggs used for control</th>
<th>Date</th>
<th>Test for haemagglutinating agents</th>
<th>Result directly</th>
<th>Result after one passage</th>
<th>Other tests performed</th>
</tr>
</thead>
</table>

#### Single harvests

<table>
<thead>
<tr>
<th>Antibiotics used during preparation, if any</th>
</tr>
</thead>
</table>

#### Tests of neutralized virus pool in animals

<table>
<thead>
<tr>
<th>Date of single harvest</th>
<th>No. of single harvest</th>
</tr>
</thead>
</table>
### Live Influenza Vaccine

**Total volume**

**Storage conditions of samples**

<table>
<thead>
<tr>
<th>Test in adult mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
</tr>
<tr>
<td>Date of inoculation</td>
</tr>
<tr>
<td>Observation period</td>
</tr>
<tr>
<td>Results</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test in suckling mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
</tr>
<tr>
<td>Date of inoculation</td>
</tr>
<tr>
<td>Observation period</td>
</tr>
<tr>
<td>Results</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Test in human cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
</tr>
<tr>
<td>Type of cells</td>
</tr>
<tr>
<td>Observation period</td>
</tr>
<tr>
<td>Results</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test in simian cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
</tr>
<tr>
<td>Type of cells</td>
</tr>
<tr>
<td>Observation period</td>
</tr>
<tr>
<td>Results</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test in chicken cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
</tr>
<tr>
<td>Type of cells</td>
</tr>
<tr>
<td>Observation period</td>
</tr>
<tr>
<td>Results</td>
</tr>
</tbody>
</table>
### Live Influenza Vaccine

#### Test in embryonated eggs

<table>
<thead>
<tr>
<th>Volume tested</th>
<th>No. of eggs inoculated by the allantoic route</th>
<th>No. of inoculated yolk sacs</th>
<th>Results</th>
</tr>
</thead>
</table>

#### Sterility test

<table>
<thead>
<tr>
<th>Results</th>
</tr>
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</table>

#### Test for mycobacteria

<table>
<thead>
<tr>
<th>Method</th>
<th>Results</th>
</tr>
</thead>
</table>

**Bulk Suspension**

<table>
<thead>
<tr>
<th>Method of clarification</th>
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</table>

<table>
<thead>
<tr>
<th>Date of preparation</th>
<th>Storage conditions of samples</th>
<th>Virus content</th>
<th>Date of test</th>
<th>Results of egg titration</th>
</tr>
</thead>
</table>

**Final Bulk**

<table>
<thead>
<tr>
<th>Date of preparation</th>
<th>Substances added</th>
</tr>
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</table>

**Final product**

<table>
<thead>
<tr>
<th>Identity test</th>
<th>Date</th>
<th>Method</th>
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</thead>
</table>

<table>
<thead>
<tr>
<th>Results:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemagglutinin</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td></td>
</tr>
<tr>
<td>Markers of attenuation</td>
<td></td>
</tr>
</tbody>
</table>

| Sterility test               |   |
| Date                         |   |
| Results                      |   |

| Virus content                |   |
| Date                         |   |
| No. of ampoules tested       |   |
| Type A, EID_{50}/ml found in each ampoule |   |
| Type B, EID_{50}/ml found in each ampoule |   |

| Inocuity tests               |   |
| Date                         |   |
| No. of mice given injections |   |
| Volume and route             |   |
| Observation period           |   |
| Results                      |   |
| No. of guinea-pigs           |   |
| Volume and route             |   |
| Observation period           |   |
| Results                      |   |

| Residual moisture            |   |
| Size of sample               |   |
| Moisture content (%)         |   |

Signature of head of laboratory

---

---
Certification by person taking overall responsibility for production of the vaccine:
I certify that lot No. ___ of vaccine satisfies Part A of the WHO Requirements for Influenza Vaccine (Live).

Signature

Name typed

The protocol must be accompanied by a sample of the label and a copy of the leaflet.

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The Revised Requirements for Influenza Vaccine (Inactivated) and the Requirements for Influenza Vaccine (Live) were prepared by the following WHO consultants and staff members:

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REFERENCES

Annex 4

BIOLOGICAL SUBSTANCES:
INTERNATIONAL STANDARDS, REFERENCE PREPARATIONS, AND REFERENCE REAGENTS

A list of international biological standards, international biological reference preparations, and international biological reference reagents previously included as annexes to the reports of the WHO Expert Committee on Biological Standardization is issued as a separate publication, revised from time to time. The most recent list\(^1\) includes all changes and additions accepted by the WHO Expert Committee on Biological Standardization in 1976, 1977, and 1978 (twenty-eighth, twenty-ninth, and thirtieth reports). Copies of the list may be obtained direct (or through booksellers) from the agents shown on the back cover of this report, or they may be ordered from: World Health Organization, Distribution and Sales Service, 1211 Geneva 27, Switzerland.

Annex 5

REQUIREMENTS FOR BIOLOGICAL SUBSTANCES
AND OTHER SETS OF RECOMMENDATIONS

The specification of requirements to be fulfilled by preparations of biological substances is necessary in order to ensure that these products are safe, reliable, and potent prophylactic or therapeutic agents. International recommendations on requirements are intended to facilitate the exchange of biological substances between countries and to provide guidance to workers responsible for the production of these substances as well as to others who may have to decide upon appropriate methods of assay and control.

Recommended requirements and sets of recommendations concerned with biological substances formulated by international groups of experts and published in the WHO Technical Report Series are listed hereunder.

<table>
<thead>
<tr>
<th>No.</th>
<th>Year</th>
<th>Requirements for Biological Substances:</th>
</tr>
</thead>
<tbody>
<tr>
<td>178</td>
<td>1959</td>
<td>1. General Requirements for Manufacturing Establishments and Control Laboratories&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Requirements for Poliomyelitis Vaccine (Inactivated)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>179</td>
<td>1959</td>
<td>3. Requirements for Yellow Fever Vaccine&lt;sup&gt;1&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>4. Requirements for Cholera Vaccine&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>180</td>
<td>1959</td>
<td>5. Requirements for Smallpox Vaccine&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>200</td>
<td>1960</td>
<td>6. General Requirements for the Sterility of Biological Substances&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>237</td>
<td>1962</td>
<td>7. Requirements for Poliomyelitis Vaccine (Oral)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> Refer to subsequent revised requirements.
274 1964 WHO Expert Committee on Biological Standardization:
   8. Requirements for Pertussis Vaccine
   9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate

293 1964 WHO Expert Committee on Biological Standardization:
   10. Requirements for Diphtheria Toxoid and Tetanus Toxoid

323 1966 WHO Expert Group:
   Requirements for Biological Substances (Revised 1965)
   1. General Requirements for Manufacturing Establishments and Control Laboratories
   2. Requirements for Poliomyelitis Vaccine (Inactivated)
   7. Requirements for Poliomyelitis Vaccine (Oral)
   5. Requirements for Smallpox Vaccine

329 1966 WHO Expert Committee on Biological Standardization:
   11. Requirements for Dried BCG Vaccine
   12. Requirements for Measles Vaccine (Live) and Measles Vaccine (Inactivated)

361 1967 WHO Expert Committee on Biological Standardization:
   13. Requirements for Anthrax Spore Vaccine (Live—for Veterinary Use)
   14. Requirements for Human Immunoglobulin
   15. Requirements for Typhoid Vaccine
   9. Requirements of Procaine Benzylpenicillin in Oil with Aluminium Monostearate (Revisions adopted 1966)

384 1968 WHO Expert Committee on Biological Standardization:
   16. Requirements for Tuberculins
   17. Requirements for Inactivated Influenza Vaccine

413 1969 WHO Expert Committee on Biological Standardization:
   4. Requirements for Cholera Vaccine (Revised 1968)
   18. Requirements for Immune Sera of Animal Origin

444 1970 WHO Expert Committee on Biological Standardization:
   19. Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)
   20. Requirements for Brucella abortus Strain 19 Vaccine (Live—for Veterinary Use)

Development of a National Control Laboratory for Biological Substances (a guide to the provision of technical facilities)

---

2 Refer to subsequent revised requirements.
3 Refer also to subsequent addendum.
WHO Expert Committee on Biological Standardization:

21. Requirements for Snake Antivenins

WHO Expert Committee on Biological Standardization:

7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1971)

WHO Expert Committee on Biological Standardization:

4. Requirements for Cholera Vaccine (Revised 1968) (Addendum 1973)
6. General Requirements for the Sterility of Biological Substances (Revised 1973)
17. Requirements for Inactivated Influenza Vaccine (Addendum 1973)
22. Requirements for Rabies Vaccine for Human Use

WHO Expert Committee on Biological Standardization:

Recommendations for the Assessment of Binding Assay Systems (including Immunoassay and Receptor Assay Systems) for Human Hormones and their Binding Proteins (a guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytochemical bioassay systems)
Development of national assay services for hormones and other substances in community health care

WHO Expert Committee on Biological Standardization:

3. Requirements for Yellow Fever Vaccine (Revised 1975)
20. Specifications of tests used in the Requirements for Brucella abortus Strain 19 Vaccine (Live—for Veterinary Use) (Addendum 1975)

WHO Expert Committee on Biological Standardization:

23. Requirements for Meningococcal Polysaccharide Vaccine (Addendum 1976)\(^4\)
24. Requirements for Rubella Vaccine (Live)
25. Requirements for Brucella melitensis Strain 1 Vaccine (Live—for Veterinary Use)

WHO Expert Committee on Biological Standardization:

27. Requirements for the Collection, Processing and Quality Control of Human Blood and Blood Products
23. Requirements for Meningococcal Polysaccharide Vaccine (Addendum 1977, incorporating Addendum 1976)
17. Requirements for Inactivated Influenza Vaccine (Addendum 1977)
Guidelines for the Preparation and Establishment of Reference Materials for Biological Substances

\(^4\) Refer also to subsequent addendum.

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<table>
<thead>
<tr>
<th>No.</th>
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<td>638</td>
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<td>WHO Expert Committee on Biological Standardization:</td>
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<tr>
<td></td>
<td></td>
<td>8 and 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Revised 1978)</td>
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<td></td>
<td></td>
<td>11. Requirements for Dried BCG Vaccine (Revised 1978)</td>
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<tr>
<td></td>
<td></td>
<td>17. Requirements for Influenza Vaccine (Inactivated) (Revised 1978)</td>
</tr>
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<td>28. Requirements for Influenza Vaccine (Live)</td>
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