WHO Expert Committee on Biological Standardization

Thirty-first Report

World Health Organization
Technical Report Series
658

World Health Organization, Geneva 1981
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PRINTED IN SWITZERLAND
80/4889 – Schäfer SA – 6500
WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Thirty-first Report

Annex 5

REQUIREMENTS FOR POLIOMYELITIS VACCINE (ORAL)

(Requirements for Biological Substances No. 7)
(Revised 1971)

Addendum 1980

CORRIGENDUM

Page 170, lines 4–5

Delete: ... Stored at 40°C, the decanted medium may be used for further subcultivation of cells.

Insert: ... Stored at 4°C, the decanted medium may be used for further subcultivation of cells.
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Geneva, 15–22 April 1980

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WHO EXPERT COMMITTEE ON
BIOLOGICAL STANDARDIZATION

Thirty-first Report

GENERAL

The WHO Expert Committee on Biological Standardization met in Geneva from 15 to 22 April 1980. The meeting was opened on behalf of the Director-General by Dr V. Fattorusso, Director, Division of Prophylactic, Diagnostic, and Therapeutic Substances.

The Committee has been unwilling for many years to set up biological standards for antibiotics used in the treatment of malignant tumours when the only known way of measuring their biological activity is a microbiological assay not involving mammalian cells, particularly for antitumour antibiotics that are complex mixtures of related biologically active components—e.g., bleomycin. However, with the development of analytical techniques that have allowed the composition of a given preparation to be measured precisely and the variability in composition of different batches to be maintained within acceptable limits, it was considered that an international reference material for bleomycin (bleomycin complex $A_2/B_2$) would serve a useful purpose. The International Reference Preparation of Bleomycin has now been established and it will be used for standardization in microbiological assay. Accelerated degradation studies of the International Reference Preparation of Bleomycin have demonstrated, however, that the reservations of the Committee on the value of microbiological assays of antitumour antibiotics were soundly based. Potencies of bleomycin preparations estimated by microbiological assays in terms of the international unit of activity are likely to provide relative measures of antitumour activity only if the component composition of each preparation has been shown by chemical analysis to be within acceptable limits. Such limits are included in Guidelines for the Quality Assessment of Antitumour Antibiotics adopted by the Committee.

These guidelines are intended as an interim measure until definitive international specifications for antitumour antibiotics are adopted. National authorities in countries that import antitumour antibiotics and their dosage forms should find the guidelines helpful in examining and judging the quality of batches of these materials. The guidelines provide information on the characteristic chemical, physical, and bio-
logical properties of 11 antitumour antibiotics and their preparations. The control information included in the guidelines was collected from published national specifications, from unpublished data provided to WHO, and from the scientific literature. References to such sources are given. Since many of the tests require the use of an authenticated sample for qualitative comparison or a reference preparation with a defined activity for biological assays of potency, existing national reference materials are listed. Where authentic material is not available for infrared identification tests, spectra of authentic samples are included.

The heterogeneity of other antibiotics such as neomycin and bacitracin also causes problems of discrepancy between potency estimates in different laboratories. As the antibacterial activity measured in these assays is more closely related to the desired therapeutic activity, the possible complications of such discrepancies are less important than those for an antitumour antibiotic such as bleomycin for which assayed activity and therapeutic activity are less similar. However, the combined use of chemical analysis of component composition and microbiological assay of potency is the only satisfactory quality control procedure for all heterogeneous antibiotic complexes.

The Committee recognized the achievement of introducing uniformity into the practices used in the production and testing of blood and plasma products. Blood and plasma components must eventually be obtained from national communities and their supply cannot continue to be wholly dependent on imports. It is imperative to formulate further requirements for blood components, for specialized plasma collection by plasmapheresis, and for the products that can be derived from such plasma. To this end the Committee stressed the importance of the cooperation of WHO with other international organizations to continue to promote the training of personnel engaged in blood collection, the separation of blood components, and quality control.

The Committee observed that the control of oral anticoagulant therapy (widely used in the treatment and prophylaxis of thrombotic disorders) requires the performance of a blood test (prothrombin time) which is based on the use of certain tissue extracts called thromboplastins. Various types of thromboplastins are prepared in different countries, and to enable the results of prothrombin time tests made with such reagents to be related to each other it is necessary to characterize the thromboplastins with materials using a particular procedure. Thus three International Reference Preparations of Human, Bovine, and Rabbit Thromboplastins respectively have been estab-
lished. These reference preparations allow more precise calibrations to be made of thromboplastins from different tissue sources. The Committee adopted the Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy, which describes the procedures involved in the preparation, testing and calibration of thromboplastin products from different tissue sources. The Committee agreed that the use of calibrated thromboplastins and the use of a detailed procedure to be followed in calibrating thromboplastins, together with a suggested method for reporting the results of prothrombin time tests, could considerably improve the overall quality of oral anticoagulant therapy.

The Committee adopted also Requirements for Hepatitis B Vaccine. This was the first time that requirements had been formulated for a biological product before it had been licensed in any country. The Committee agreed, however, that in view of the difficulties in inactivating the virus while preserving immunogenicity, some methods currently being investigated may not be successful in yielding products that are consistently safe and potent. Unfortunately, the virus has not yet been grown in cell cultures and the animal most sensitive to the virus is still the chimpanzee. As these animals are difficult to obtain, some investigators might be tempted to place on clinical trial a product that might not have been adequately tested for safety, and it was this danger to human health that the Committee wished to prevent. It was agreed that even though the Requirements could not be specific in every particular, they would be useful for control authorities as a basis for their national requirements for the protection of their communities.

The Committee adopted Guidelines for the National Control of Vaccines and Sera that will assist developing countries in establishing control activities. These guidelines approach the setting up of quality control in three phases of development, from the limited activity of evaluating manufacturers' protocols to the most complex activity involved in running a fully comprehensive laboratory facility. The important objective is to make freely available the necessary technology so that every country will have the opportunity of effecting some control of the vaccines and sera used within that country, which is of importance to prevent hazards to health.

The Committee agreed that there was an urgent need for more information on the stability of vaccines, particularly when they are to be used in countries with high ambient temperatures and where there may also be severe limitations on refrigeration facilities during transport and storage (the cold chain). The Committee was informed that
much attention is being directed to the preparation of more stable vaccines, and it agreed that it would be useful to have special reference materials for the assessment of stability at higher temperatures so that manufacturers could compare the stabilities of their products with those of the special materials. The Committee also considered that it would be useful to include a test for stability at higher temperatures in the requirements for each vaccine.

The Committee noted with interest the report of the WHO Coordination Meeting on Venoms and Antivenoms and agreed that this comprehensive document (WHO Offset Publication No. 58) would be useful in many countries, especially in those in which snake bites are frequent and cause death. The Committee agreed also that the collection of venoms from the seven species of snakes that are the greatest danger, and the preparation and standardization of their antivenoms, could result in a marked improvement of the present situation.

With the steadily increasing range and number of \textit{in vitro} tests carried out in clinical pathology, there is a trend towards the use of kits of matched reagents and towards automation. Clinical endocrinology, for example, relies extensively on hormone estimations using immunoassay kits, and pregnancy-testing kits based on the detection of human chorionic gonadotrophin are now widely used by the public. Almost all such tests employ biological reagents and require reference materials and meticulous standardization. Since 1974, when the Committee formulated its Recommendations for Immunoassay Kits, sufficient experience has been gained to enable Requirements for Immunoassay Kits, incorporating Guidelines for Assessing the Performance of Immunoassay Kits for Health Care, to be formulated and adopted. In haematology, automated methods (such as nephelometry) for the determination of serum proteins and blood groups and for the screening of blood donations (especially for anti-D antibodies) are now established routine procedures. The Committee agreed that it is becoming necessary to include such automated assay procedures in collaborative studies to evaluate the suitability of a proposed standard.

Similar trends towards the use of kits and automation occur also in tests used in clinical immunopathology, disorders of haemostasis, and clinical enzymology: in all such areas standardization, guidelines, and appropriate reference materials are required.

The formulation of the Requirements for Immunoassay Kits highlighted many aspects of assay standardization that still require definition and agreement. In collaboration with the International Atomic
Energy Agency, the International Federation of Clinical Chemistry, and the International Society of Endocrinology, proposals are being formulated on the nature and classification of protein-binding assays, on the terminology used in assay methodology, and on the quality assessment of the performance of such tests. One fundamental question requiring an answer is what distinction there is (if any) between a bioassay and a protein-binding assay. For example, agreement on the identity of what is called chorionic gonadotrophic hormone—and hence on the nature of materials to be used for international biological standards—depends on an answer to this question. Traditionally a "hormone" is identified by its activity in one or more characteristic biological assays, and each such activity is quantified by comparison with a standard. Thus the material used as the standard and the bioassay system(s) employed together define what is called "the hormone".

With the introduction of analogues of peptides (such as desmopressin) made by chemical synthesis and particularly of protein hormones (such as insulin and growth hormone) using recombinant DNA technology, the detailed characterization of the pattern of their properties in various biological systems will form an essential part of the identification of such products intended for sustained replacement therapy.

The Committee noted that the large-scale production of biological proteins by recombinant DNA has advanced to such an extent that a number of biological products for use in medical treatment (e.g., insulin, interferon, growth hormone, and somatostatin) may soon be made in significant quantity in this way. The control of the identity, purity, potency, and safety of such products for medical use may present new problems. The Committee agreed that in view of the far-reaching implications of these new developments, relevant technical data should be collected by WHO and guidelines for the control of such substances prepared.
SUBSTANCES

ANTIBIOTICS

1. Streptomycin

The Committee noted the report of the collaborative assay referred to in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 12; WHO/BS/80.1273). The Committee noted also that, in accordance with the authorization in its twenty-ninth report (WHO Technical Report Series, No. 626, 1978, p. 10), the National Institute for Biological Standards and Control, London, had established the third International Standard for Streptomycin and, on the basis of the results of the collaborative assay and with the agreement of the participants, had defined the activity of the contents of each ampoule of the International Standard for Streptomycin as 78 500 International Units of Streptomycin.

2. Amikacin

The Committee noted that a portion of the preparation of amikacin referred to in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 12) had been freeze-dried in ampoules (WHO/BS/80.1274). The Committee was informed that the freeze-dried material had satisfactory stability, that the remainder of the material would be freeze-dried under identical conditions, and that a collaborative assay would be arranged. The Committee authorized the National Institute for Biological Standards and Control, London, to establish the preparation as the international reference preparation of amikacin, and, on the basis of the results of the collaborative assay and with the agreement of the participants, to define the international unit.

3. Sisomicin

The Committee noted that, in accordance with the request in its twenty-ninth report (WHO Technical Report Series, No. 626, 1978,

References prefixed “WHO/BS” are to unpublished working documents obtainable on request from Biologicals, World Health Organization, Geneva, Switzerland.
p. 12), a quantity of sisomicin sulfate had been obtained (WHO/BS/80.1275) and that a small portion of this material had been dispensed into ampoules and freeze-dried. The Committee was informed that if tests show this preparation to be stable the remaining material will be freeze-dried under identical conditions and a collaborative assay arranged.

4. Tobramycin


In view of the need for an international reference preparation of tobramycin the Committee established the preparation studied as the International Reference Preparation of Tobramycin and defined the International Unit of Tobramycin as the activity contained in 0.0010142 mg of the International Reference Preparation of Tobramycin.

The Committee noted that difficulties had been encountered in the collaborative assay in handling the material owing to its extremely hygroscopic nature and the fragility of the ampoules. The Committee therefore requested the National Institute for Biological Standards and Control, London, to issue with the ampoules a memorandum emphasizing these problems and making recommendations to minimize them.

The Committee also requested the National Institute for Biological Standards and Control to investigate the possibility of preparing material freeze-dried in ampoules, as a replacement for the international reference preparation.

5. Bleomycin (Bleomycin Complex A₂/B₂)

The Committee noted the results (WHO/BS/80.1276) of the collaborative assay of the preparation of bleomycin complex A₂/B₂ referred to in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 13). The Committee noted also that the preparation had a composition typical of material used for therapy and had adequate stability. The Committee therefore established the preparation
as the International Reference Preparation of Bleomycin and defined the activity of the contents of each ampoule as 8910 International Units of Bleomycin.

6. **Bleomycin A₅**

The Committee was informed that the National Institute for Biological Standards and Control, London, had received only a single sample of bleomycin A₅, from a batch being used clinically, and that, in accordance with the request in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 13), further samples were being sought.

**ANTIBODES**

7. **Anti-Toxoplasma Serum, Human**

The Committee noted that stocks of the International Standard for Anti-Toxoplasma Serum, Human, were becoming low (WHO/BS/80.1277). The Committee noted also that a replacement preparation had been obtained and that an international collaborative assay had been carried out, the results of which showed that the replacement preparation was suitable to serve as an international standard. The Committee therefore established the preparation as the second International Standard for Anti-Toxoplasma Serum, Human, and, on the basis of the results of the collaborative assay, defined the activity of the contents of each ampoule as 2000 International Units of Anti-Toxoplasma Serum, Human.

8. **Clostridium botulinum Type B Antitoxin**

The Committee noted that the preparation of *Clostridium botulinum* type B antitoxin referred to in its twenty-ninth report (WHO Technical Report Series, No. 626, 1978, p. 13) had been processed and freeze-dried in ampoules (WHO/BS/80.1289). The Committee was informed that the international collaborative assay referred to in its twenty-eighth report (WHO Technical Report Series, No. 610, 1977, p. 19) would now be arranged by the State Serum Institute, Copenhagen.
9. Antivenoms

The Committee noted the report of a WHO meeting held to coordinate work on the use and standardization of venoms and antivenoms (WHO/BS/80.1292). The Committee was informed that the limited availability of antivenoms was of considerable concern, particularly in developing countries. The Committee noted also that the experts had reviewed the world situation and had identified seven species of snakes as causing the greatest problems.

The Committee agreed that it would be desirable to have standards for antivenoms against the venoms of these seven species and requested WHO to take the necessary steps to establish these. In view of the widespread interest in this subject an account of recent progress in the standardization of venoms and antivenoms, based on the report of the above-mentioned meeting, was being issued as WHO Offset Publication No. 58.

ANTIGENS

10. Purified Protein Derivative (PPD) of Bovine Tuberculin

The Committee noted (WHO/BS/80.1282) that estimates of the potency of PPD of bovine tuberculin relative to the International Standard for PPD of Mammalian Tuberculin (Human) differ depending on whether the guinea-pigs used in the laboratory assay are sensitized with heat-killed Mycobacterium bovis or with live BCG vaccine and on whether the assay is performed with cattle infected with M. bovis.

The Committee agreed that, before an international collaborative assay was carried out, more work was necessary both in the laboratory and with infected cattle to compare the proposed international standard for bovine PPD with a number of national standards for bovine PPD and the International Standard for PPD of Mammalian Tuberculin (Human).

The Committee requested the Central Veterinary Laboratory, Weybridge, England, to proceed with the international collaborative assay should the proposed standard, on completion of these tests, be confirmed as suitable.
11. Purified Protein Derivative (PPD) of Mallein

The Committee noted (WHO/BS/80.1283) that, in accordance with the request in its twenty-ninth report (WHO Technical Report Series, No. 626, 1978, p. 16), the Central Veterinary Laboratory, Weybridge, England, had obtained four preparations of mallein PPD and had carried out preliminary tests in guinea-pigs and rabbits. The Committee was informed that, if further tests of these preparations showed one or more of them to be suitable to serve as the international standard, the Central Veterinary Laboratory would arrange an international collaborative assay.

12. Clostridium botulinum Type B Toxin

The Committee noted that, in accordance with the request in its twenty-seventh report (WHO Technical Report Series, No. 594, 1976, p. 16), the State Serum Institute, Copenhagen, in conjunction with the Chiba Serum Institute, Chiba, Japan, had completed the studies of the heterogeneity of the Clostridium botulinum type B toxin (WHO/BS/80.1290). The results of the studies showed that the strain QC, from which the unusual toxin was obtained, is an atypical strain of Clostridium botulinum type B. Moreover, it is possible to select bleedings from horses immunized with toxoids from typical Clostridium botulinum type B so that the atypical strain does not reveal the heterogeneity of its toxin in neutralization tests using the International Standard for Clostridium botulinum type B Antitoxin.

The Committee agreed that the probability of the occurrence of a toxin from strains such as QC would be infrequent and unimportant in the calibration of Clostridium botulinum type B antitoxin. The Committee agreed, therefore, that for this purpose no further work on such toxin was justified.

13. Diphtheria and Tetanus Toxoids for Flocculation Tests

The Committee noted the results of an inquiry concerning the need for international reference materials for diphtheria and tetanus toxoids for use in the titration of the activity of toxoids in the floccu-
lation test (WHO/BS/80.1253). The Committee noted also that most of the laboratories favoured international reference toxoids being made available for this purpose.

These international reference materials would be particularly useful in developing countries that are establishing quality control procedures. However, it would be necessary to have such toxoids available as reference preparations without an assigned unitage. The Committee emphasized that international reference preparations of corresponding antitoxins to these materials had been established for the flocculation test and LF equivalents had been defined for them. In order to clarify this issue the Committee requested WHO to take the necessary steps to determine whether reference reagent toxoids would be useful in practice.

14. Live Newcastle Disease Vaccines

The Committee noted (WHO/BS/79.1247 and WHO/BS/79.1248) that the Central Veterinary Laboratory, Weybridge, England, had shown that the International Reference Preparation of Newcastle Disease Vaccine (Live), which was established primarily to standardize the measurement of the virus content of B1 and similarly attenuated Newcastle disease vaccines, could also be used to standardize the estimation of the immunogenic potency of such vaccines. The Committee requested the Central Veterinary Laboratory to arrange an international collaborative study to investigate further the suitability of the International Reference Preparation of Newcastle Disease Vaccine (Live) for this purpose.

15. Tetanus Toxin

The Committee noted that studies at the National Institute for Biological Standards and Control, London, to find a single tetanus toxin suitable for the measurement of antitoxin levels in antisera as mentioned in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 19) continued to give an irregular pattern of results (WHO/BS/80.1279). The Committee noted also that the National Institute for Public Health, Bilthoven, Netherlands, had offered to
subject the preparation of toxin studied to tests other than those of
the neutralization test in mice, to determine whether or not more
uniform results could be obtained. The Committee agreed that the
results of such studies would be useful.

16. Pertussis Vaccine

The Committee noted the report (WHO/BS/80.1288) on the col-
laborative assay of the proposed second international standard for
pertussis vaccine requested in its twenty-ninth report (WHO Technical

The Committee established the preparation studied as the second
International Standard for Pertussis Vaccine and, on the basis of the
results of the collaborative assay, defined the activity of the contents
of each ampoule as 46 International Units of Pertussis Vaccine.

17. Yellow Fever Vaccine

The Committee noted that a WHO working group had reviewed
and recommended revision of the procedure for granting WHO ap-
proval of yellow fever vaccine used in relation to the international
certificates of vaccination (WHO/BS/80.1269). The Committee
agreed that the recommended revised procedure was in accordance
with current technology and had taken into account the revisions in
the Requirements for Yellow Fever Vaccine since they were first
formulated in 1959 (WHO Technical Report Series, No. 179, 1959,
Annex 1).

The Committee adopted the Recommended Revised Procedure for
Approval by WHO of Yellow Fever Vaccines in connexion with the
Issue of International Certificates of Vaccination and agreed that it
should be annexed to this report (see Annex 1).

The Committee noted also that, in accordance with the request in
its thirtieth report (WHO Technical Report Series, No. 638, 1979,
p. 19), the National Institute for Biological Standards and Control,
London, had obtained a preparation suitable for use in the control of
the virus content of yellow fever vaccines and that an international
collaborative assay was being arranged (WHO/BS/80.1280).
18. Plasmin

The Committee noted that when the International Reference Preparation of Plasmin was established at its twenty-eighth meeting (WHO Technical Report Series, No. 610, 1977, p. 34), it was reported that the material, which was a liquid preparation, was unstable at or above 20°C (WHO/BS/80.1258). The Committee noted also that another preparation of plasmin had been distributed into ampoules and freeze-dried and had been shown by accelerated degradation studies to have much improved stability. The Committee was informed that an international collaborative study of this freeze-dried plasmin was in progress, coordinated by the National Institute for Biological Standards and Control, London.


The Committee noted that the second International Standard for Factor VIII, Human, which is a concentrate of antihaemophilic factor VIII, while suitable for the assay of factor VIII concentrates, is less suitable for the assay of this clotting factor in human plasma (WHO/BS/80.1267).

The Committee noted also that the National Institute for Biological Standards and Control, London, had obtained a proposed reference plasma that would be assayed against samples of fresh normal plasma for the content of factor VIII clotting activity (factor VIII: C) and other activities related to factor VIII.

The Committee agreed that it would be advantageous to have two reference preparations available for factor VIII assay, depending on whether the material to be assayed was normal plasma or a factor VIII concentrate. Accordingly the Committee requested the National Institute for Biological Standards and Control to coordinate an international collaborative study on the proposed reference plasma.

20. Anti-B Blood Typing Serum, Human

The Committee noted that the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, had analysed the results of the international collaborative study on the proposed
second international standard for anti-B blood typing serum, human (WHO/BS/80.1293).

The Committee established the preparation as the second International Standard for Anti-B Blood Typing Serum, Human, and defined the activity of the contents of each ampoule as 205 International Units of Anti-B Blood Typing Serum.

The Committee noted also that a further 3.5 litres of anti-B blood typing serum had been collected and was being studied for its suitability to serve as a future replacement standard (WHO/BS/80.1285).


The Committee noted that the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, had selected 3.5 litres of each of anti-A and anti-A,B sera for their specificity as blood typing sera, human (WHO/BS/80.1285). These sera had been distributed into ampoules and freeze-dried, and the suitability and potency of each of them were being assessed in an international collaborative study.

The Committee noted also that 1.5 litres of each of anti-C, anti-D, and anti-E saline reactive sera had been collected. The Committee was informed that these sera had been freeze-dried in ampoules and would be subjected to an international collaborative study.

22. Blood Group Substances A and B

The Committee noted that, in response to the request in its twenty-ninth report (WHO Technical Report Series, No. 626, 1978, p. 17), the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, had ascertained that there was no need for international reference materials for the control of blood group substances A and B that may be present in preparations of human albumin (WHO/BS/80.1284). However, since preparations of the substances are required for the immunization of donors for the provision of blood typing sera, the Committee requested the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service to obtain suitable material that could serve as a reference preparation as soon as the requirements for such immunization procedures had been defined.
23. Human Serum Immunoglobulin E

The Committee noted that the first International Reference Preparation of Human Serum Immunoglobulin E had been shown to contain hepatitis B antigen (WHO/BS/79.1240).

The Committee noted also that the National Institute for Biological Standards and Control, London, had prepared a replacement reference preparation of immunoglobulin E that had been shown to be free from hepatitis B antigen. The Committee noted further that the results of an international collaborative assay had shown the material to be stable and suitable to serve as an international reference material.

The Committee therefore established the preparation as the second International Reference Preparation of Human Serum Immunoglobulin E and, on the basis of the results of the collaborative assay, defined the activity of the contents of each ampoule as 5000 International Units of Immunoglobulin E.

24. Anti-Hepatitis A Immunoglobulin

The Committee noted that, in accordance with the request made in its twenty-ninth report (WHO Technical Report Series, No. 626, 1978, p. 18), the Bureau of Biologics, Food and Drug Administration, Bethesda, MD, USA, had arranged an international collaborative study of a proposed international reference preparation of anti-hepatitis A immunoglobulin (WHO/BS/80.1252). The Committee noted also that 17 laboratories had taken part in the study to determine its suitability as a reference material of anti-hepatitis A immunoglobulin.

25. Human Serum Complement Components Clq, C4, C5, and Factor B

The Committee noted the results of an international collaborative assay, carried out by the International Union of Immunological Societies, of the antigenic and haemolytic activities of components of complement in four preparations of sera (WHO/BS/80.1281).

On the basis of the results of the international collaborative assay, the Committee adopted and established preparation 4 as the International Reference Preparation of Human Serum Complement Components Clq, C4, C5, and Factor B and defined the activity of the
contents of each ampoule as 100 International Units of each of these four components.

26. International Reference Preparation of Haemiglobincyanide

The Committee noted that the International Reference Preparation of Haemiglobincyanide, prepared and distributed by the National Institute for Public Health, Bilthoven, Netherlands, had been replaced on 10 occasions since the first Reference Preparation of Haemiglobincyanide was established by the Committee in 1967 (WHO Technical Report Series, No. 384, 1968, p. 10). As this is a working reference preparation distributed in thousands of ampoules each year it is understandable that it needs to be replaced at frequent intervals. The Committee noted also that at the time of each replacement a limited collaborative assay had shown the new material to be suitable as a reference preparation.

The Committee was informed of a proposal that in future each successive replacement preparation would be identified by a number starting with the second International Reference Preparation of Haemiglobincyanide, distributed in January 1980 (WHO/BS/79.1243 and WHO/BS/79.1243 Add. 1). The Committee agreed that the proposed procedure for the replacement of the successive reference preparations was acceptable.

The Committee therefore authorized the National Institute for Public Health to establish future successive replacements of the International Reference Preparation of Haemiglobincyanide on the basis of the results of limited collaborative assays and requested the Institute to provide a report on each collaborative assay to WHO.

ENDOCRINOLOGICAL AND RELATED SUBSTANCES

27. Human Growth Hormone (HGH)

The Committee noted that the National Institute for Biological Standards and Control, London, had obtained sufficient quantities of candidate materials, one of which might serve as an international standard for human growth hormone (WHO/BS/80.1261). The Committee noted also that an international collaborative study in
25 laboratories had been initiated to characterize these preparations and to calibrate one in terms of the International Standard for Growth Hormone, Bovine, for Bioassay.

28. Corticotrophin, Porcine

The Committee noted (WHO/BS/80.1256) the results of thermal degradation studies on samples of the third International Standard for Corticotrophin, Porcine, for Bioassay, carried out at the National Institute for Biological Standards and Control, London, which have confirmed the stability of this standard established in 1962 (WHO Technical Report Series, No. 259, 1963, p. 13). Observations on the samples degraded at higher temperatures indicate the nature of the correlation between physicochemical alterations of the peptides present in the material and biological activity in various corticotrophin bioassay procedures.

29. Chorionic Gonadotrophin, Human

The Committee was informed that a number of manufacturers of pregnancy test kits had found difficulty in the use, for the calibration of such kits, of the International Reference Preparation of Chorionic Gonadotrophin, Human, for Immunoassay, established at its thirtieth meeting (WHO Technical Report Series, No. 638, 1979, p. 27), which consists of highly purified hormone. The Committee noted the earlier reports (WHO/BS/79.1191 and WHO/BS/78.1235) that certain kits also detected other proteins characteristically found in the urine of women in early pregnancy.

The Committee was informed also that at a meeting of WHO staff with some manufacturers of such kits, the manufacturers had agreed that they would collect a large amount of first trimester pregnancy urine and freeze-dry it in a form suitable to be distributed as a working reference material for the calibration of the kits. The Committee was informed that all stages of the processing of the material, as well as the design of the collaborative assay to assess its suitability, would be carried out in collaboration with the National Institute for Biological Standards and Control, London.
In the light of these considerations, the Committee agreed that, in the meantime, manufacturers could continue to calibrate such pregnancy test kits in terms of the unit defined by the second International Standard for Chorionic Gonadotrophin, Human, for Bioassay.

30. Pituitary Gonadotrophins FSH and LH (ICSH), Human, for Bioassay

The Committee noted that the stock of the batch of ampoules constituting the first International Reference Preparation of Pituitary FSH and LH, Human, for Bioassay, established in 1974 (WHO Technical Report Series, No. 565, 1975, p. 12) was now almost exhausted (WHO/BS/80.1255). The Committee noted also that another batch of ampoules had been prepared from the master ampoules of the same bulk preparation using a procedure identical to that used for the first International Reference Preparation of Pituitary Gonadotrophins FSH and LH (ICSH), Human, for Bioassay. The Committee agreed that the results of the bioassays showed that the potencies of the FSH and LH activities contained in the second batch of ampoules did not differ significantly from those present in ampoules of the first reference preparation. The Committee therefore established the new batch of ampoules as the second International Reference Preparation of Pituitary Gonadotrophins FSH and LH (ICSH), Human, for Bioassay and defined the activity of the contents of each ampoule of the second International Reference Preparation of Pituitary Gonadotrophins FSH and LH (ICSH), Human, for Bioassay, as 10 International Units of Pituitary FSH and 25 International Units of Pituitary LH.

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

The Committee noted (WHO/BS/80.1264) that an additional preparation of human pituitary FSH had recently been obtained by the National Institute for Biological Standards and Control, London, that preliminary tests had shown that it was more pure than the preparations hitherto studied (WHO/BS/78.1221) as candidate materials for an international reference preparation for immunoassay, and that
this preparation would be included in the international collaborative study.

32. Gonadotrophin-Releasing Hormone (Gonadorelin)

The Committee noted the results of the collaborative study of a preparation of synthetic gonadorelin (WHO/BS/80.1257). The Committee noted also that bioassays by procedures used for the control of preparations of this peptide had shown that the preparation was suitable to serve as a standard in these procedures.

The Committee therefore established the preparation as the International Reference Preparation of Gonadorelin, for Bioassay, and defined the activity of the contents of each ampoule as 31 International Units of Gonadorelin.

33. Secretin

The Committee noted that, in accordance with the request made in its twenty-sixth report (WHO Technical Report Series, No. 565, 1975, p. 18), the National Institute for Biological Standards and Control, London, had obtained a quantity of a preparation of synthetic secretin.

The Committee noted also that this material had been freeze-dried in ampoules, that stability tests had shown that it was adequately stable, and that it had biological activity similar to that of a sample of the natural hormone. Since a stable preparation of this hormone is now available, the Committee requested the National Institute of Biological Standards and Control to arrange an international collaborative study.

34. Parathyroid Hormone, Human

The Committee noted that, in accordance with the request made in its twenty-sixth report (WHO Technical Report Series, No. 565, 1975, p. 9), a quantity of human parathyroid hormone extracted from adenomas had now been obtained (WHO/BS/80.1254). The Committee noted also that this very scarce material had been characterized as far as practicable and that the results of preliminary in vitro bio-
assays had shown that the freeze-dried material gave responses characteristic of the intact natural hormone.

Furthermore, the Committee noted that the National Institute for Biological Standards and Control, London, had initiated an international collaborative assay to assess the suitability of the material to serve as an international reference preparation of parathyroid hormone, human, for immunoassay.

35. Insulin

The Committee noted the proposals (WHO/BS/80.1262) for the replacement of the fourth International Standard for Insulin, Bovine and Porcine, for Bioassay, established in 1958 (WHO Technical Report Series, No. 172, 1959, p. 10), and agreed that it would be desirable to use a preparation of highly purified porcine insulin as replacement material.

The Committee also agreed that it would be desirable to obtain evidence attesting to the suitability of a standard of such material for the assay of highly purified insulin of bovine as well as of porcine origin, and of preparations of insulins currently available, including, if possible, a sample of insulin prepared by recombinant DNA procedures. The Committee therefore requested the National Institute for Biological Standards and Control, London, to obtain samples of suitable materials that could be included in the international collaborative assay for the calibration of the new standard.

36. Desmopressin

The Committee noted that, in accordance with the request made in its twenty-ninth report (WHO Technical Report Series, No. 626, 1978, p. 23), the National Institute for Biological Standards and Control, London, had obtained a preparation of purified desmopressin (WHO/BS/78.1232), which had been freeze-dried in ampoules, and that an international collaborative study had been completed (WHO/BS/80.1266).

The Committee established the preparation as the International Standard for Desmopressin and defined the activity of the contents of each ampoule as 27 International Units of Desmopressin.
REFERENCE REAGENTS

37. Pyrogens

The Committee noted that, in accordance with the request made in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 32), the National Institute for Biological Standards and Control, London, had initiated an international collaborative study on Limulidae amoebocyte lysate tests for the detection of bacterial endotoxins (WHO/BS/80.1265). The Committee agreed that the study could provide useful data on which to base future decisions concerning the applications of this test procedure.

38. Subtype Specific Antisera to Hepatitis B Surface Antigens

The Committee noted that, in addition to the panel of reference sera for the detection of both hepatitis antigen HBsAg and antibody anti-HBs to hepatitis B referred to in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 29), there was a need for specific antisera to the subtypes of hepatitis B surface antigen that were not represented in the reference reagent panels. The Committee noted also the results of specificity tests on five antisera (WHO/BS/80.1287). On the basis of these results the Committee established the five sera:

- guinea-pig anti-HBs/ad serum
- guinea-pig anti-HBs/ay serum
- rabbit anti-HBs/ar serum
- goat anti-HBs/ad serum
- goat anti-HBs/ay serum

as International Reference Reagents for the detection of the specific subtypes of hepatitis B surface antigen.
39. **Requirements for Rabies Vaccine for Human Use**

The Committee noted that, in accordance with the request in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 33), the Requirements for Rabies Vaccine for Human Use (WHO Technical Report Series, No. 530, 1973, Annex 3) had been revised (WHO/BS/79.1238 Rev. 1). The Committee noted also that these revised requirements took into consideration the current technology used in the production of rabies vaccines as well as the establishment of the third International Reference Preparation of Rabies Vaccine (WHO Technical Report Series, No. 638, 1979, p. 15).

The Committee was informed that the document had not been combined with the Requirements for Rabies Vaccine for Veterinary Use because the group of consultants drafting the reports had recommended that they should be separate documents.

After making a number of amendments the Committee adopted the revised Requirements for Rabies Vaccine for Human Use and agreed that they should be annexed to this report (Annex 2).

40. **Requirements for Rabies Vaccine for Veterinary Use**

The Committee noted that, in accordance with the request made in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 33), Requirements for Rabies Vaccine for Veterinary Use had been formulated (WHO/BS/79.1237 Rev. 1).

After making a few minor modifications the Committee adopted the Requirements for Rabies Vaccine for Veterinary Use and agreed that they should be annexed to this report (Annex 3).

41. **Requirements for Hepatitis B Vaccine**

The Committee noted that Requirements for Hepatitis B Vaccine had been formulated for the preparation of safe and potent vaccines (WHO/BS/79.1239 and WHO/BS/79.1239 Add. 1). The Committee was informed that in several sections of the requirements the specifi-
cations concerning the details of the tests to ensure safety and efficacy had been left to the national control authority. The Committee was informed that for these reasons it had been suggested that the document should be designated as guidelines rather than requirements. After a thorough discussion, however, the Committee decided that since some control authorities had expressed a need for requirements the original title should be retained.

After making some amendments the Committee adopted the Requirements for Hepatitis B Vaccine and agreed that they should be annexed to this report (Annex 4).

42. Requirements for Poliomyelitis Vaccine (Oral)

The Committee was informed that since the Requirements for Poliomyelitis Vaccine (Oral) had last been revised (WHO Technical Report Series, No. 486, 1972, Annex 1) there had been a number of technical developments that concerned particularly the karyology of human diploid cells and the adoption of an approved method for the evaluation of neurotropic activity of poliomyelitis vaccines (oral). Furthermore, international reference materials for the titration of virus content have been adopted by the WHO Consultative Group on Poliomyelitis Vaccine, and work is in progress leading to the adoption of neurovirulence reference materials.

The Committee noted the proposed revisions for the Requirements for Poliomyelitis Vaccine (Oral) (WHO/BS/80.1249 and WHO/BS/80.1249 Add. 1). After making a number of amendments, and in view of the importance of obtaining international agreement, particularly on the neurovirulence test, the Committee adopted the revisions and agreed that the addendum should be annexed to this report (Annex 5).

43. Requirements for Meningococcal Polysaccharide Vaccine

The Committee noted (WHO/BS/80.1250 Rev. 1) that since the publication of the last addendum to the Requirements for Meningococcal Polysaccharide Vaccine in its twenty-ninth report (WHO Technical Report Series, No. 626, 1978, p. 25) the polysaccharides from strains of Neisseria meningitidis representative of Groups Y, 29E and W135 had been isolated and purified. Furthermore, the organisms in
Groups Y and W135 that were causing more than 10% of the cases of bacterial meningitis were particularly virulent. The Committee agreed that it would be desirable to make provision for the inclusion of these polysaccharides in the vaccine, and the requirements have therefore been amended. In order to avoid the need to refer to earlier documents in which amendments have appeared the Committee agreed that the amendments included in the twenty-ninth report should be included with the latest revision to form a cumulative addendum.

The Committee adopted the Addendum to the Requirements for Meningococcal Polysaccharide Vaccine and agreed that it should be annexed to this report (Annex 6).

44. Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy

The Committee noted that, in response to the recommendation in its twenty-eighth report (WHO Technical Report Series, No. 610, 1977, p. 51) and in accordance with the request in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 34), proposed Requirements for Thromboplastins had been reformulated (WHO/BS/77.1145 Rev. 1 and WHO/BS/77.1145 Rev. 1, Add. 1).

The Committee was informed that the reports of the collaborative studies of thromboplastins, requested in its twenty-eighth report (WHO Technical Report Series, No. 610, 1977, p. 14), would soon be made available to WHO (Thrombosis and haemostasis, 42: 1073–1114 (1979)).

The Committee noted also that there were still some problems in respect of the use of the calibration constant system, but in furtherance of the aim of standardization in this important aspect of haemostasis, it adopted the reformulated Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy and agreed that they should be annexed to this report (Annex 7).

45. Requirements for Immunoassay Kits

The Committee noted that, in accordance with the request in the general discussion at its thirtieth meeting (WHO Technical Report Series, No. 638, 1979, p. 10) and since kits for the detection and/or estimation of many different substances were now being used exten-
sively, there was a need for requirements for the control of such kits (WHO/BS/80.1270).

The Committee agreed that it was particularly necessary that (i) each component should be standardized in the system in which it was to be used, (ii) there should be reproducibility of estimates made with successive batches of the kits, and (iii) the kits should be labelled correctly and accompanied by information on their performance characteristics and application. In view of the extensive variety and wide usage of such kits the Committee agreed that the glossary of terms used in the document was an essential part of the requirements.

After making a number of amendments, the Committee adopted the Requirements for Immunoassay Kits and agreed that they should be annexed to this report (Annex 8).

46. Guidelines for Quality Assessment of Antitumour Antibiotics

The Committee noted that, in accordance with the request made in its thirtieth report (WHO Technical Report Series, No. 638, 1979, p. 33), Guidelines for the Quality Assessment of Antitumour Antibiotics had been distributed, and on the basis of comments received the final document had been prepared (WHO/BS/79.1244 and WHO/BS/79.1244 Add. 1).

After making a number of amendments the Committee adopted the Guidelines for Quality Assessment of Antitumour Antibiotics and agreed that they should be annexed to this report (Annex 9).

47. Requirements for Antibiotic Susceptibility Tests

The Committee noted that since the Requirements for Antibiotic Susceptibility Tests had been adopted (WHO Technical Report Series, No. 610, 1977, p. 22) there had still been no international agreement on the categorization of susceptibility, and that the national criteria for the evaluation of performance and the interpretation of results reproduced as examples in the requirements had recently been revised (WHO/BS/79.1245). The Committee noted also that proposals had been made to (i) revise the criteria in the requirements, (ii) change one of the three-letter codes used to identify antibiotics contained in discs, and (iii) add suggested codes for several new antibiotics (WHO/BS/79.1241).
The Committee agreed that these suggestions should be annexed to this report (Annex 10).

48. The National Control of Vaccines and Sera

The Committee noted that in order to assist further the developing countries in the organization of facilities needed in the quality control of biological substances the document “Development of a National Control Laboratory for Biological Substances” had been revised and retitled “The National Control of Vaccines and Sera” (WHO/BS/79.1246). After making a few minor amendments, the Committee adopted the revised document and agreed that it should be annexed to this report (Annex 11).

49. Requirements for Rubella Vaccine (Live)

The Committee noted that the acceptable levels of abnormalities that occur in the chromosomes of normal cells from human tissue had been revised, and that such levels should now be incorporated into the relevant section of the requirements for vaccines prepared from virus grown in human diploid cells (WHO/BS/80.1286). The Committee adopted the Addendum to the Requirements for Rubella Vaccine (Live) and agreed that it should be annexed to this report (Annex 12).

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

The Committee noted that in the Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines the test for innocuity requires a single human dose to be inoculated into mice but 5 human doses must be inoculated by the intraperitoneal route into each guinea-pig (WHO/BS/80.1295). This larger dose given by the specified route into guinea-pigs causes reactions in the animals. The Committee agreed that, since this was a test for innocuity and was additional to the tests for specific toxicity, the test in guinea-
pigs would be satisfactory if a single human dose were inoculated by
the intraperitoneal route into each of two guinea-pigs.

The Committee agreed that the addendum to these requirements
should be annexed to this report (Annex 13).

51. WHO Blood Programme

The Committee noted that the report on the WHO Blood Pro-
gramme outlined additional information that was required for the
operation of a comprehensive blood programme (WHO/BS/80.1268)
and agreed with its recommendations. The Committee also agreed
with the proposal of the WHO Secretariat to bring together specialist
groups to discuss the problems identified in the report.

ACKNOWLEDGEMENTS

The Committee thanks the following members of the WHO Sec-
retariat for their special contributions to its deliberations: Dr K. Bögel,
Veterinary Public Health; Mr P. Hall, Special Programme of Research,
Development and Research Training; Dr Lj. Higy-Mandić, Biologicals;
and Dr W. Wieniawski, Chief Pharmaceutical Officer, Pharmaceuticals.
INTRODUCTION

International certificates of vaccination or revaccination against yellow fever are valid only if the vaccine used has been approved by the World Health Organization. WHO has thus a statutory responsibility for ensuring the quality of the vaccine. The procedure at present in use for approval by WHO of yellow fever vaccine has not been revised since 1952.

Since then, changes were introduced in the WHO Requirements for Yellow Fever Vaccine in 1959 (1) and 1975 (2). It has now become necessary to update the procedure for the approval of yellow
fever vaccines by WHO. A working group was therefore convened in
Geneva from 22 to 24 May 1979 to review the situation.

1. DEVELOPMENT OF 17D YELLOW FEVER VACCINE
AND PROBLEMS INVOLVED

Wild yellow fever virus is both viscerotropic, causing liver disease,
and neurotropic. After a few intracerebral passages in mice, the virus
showed a loss of viscerotropism in rhesus monkeys, which provide an
excellent and sensitive model reflecting the disease as seen in man.
Neurotropism is retained during such passages, however, and in mice
it is enhanced, as shown by a markedly shortened incubation period.

Attempts to develop a strain of virus suitable for human vaccination
were made in the Pasteur Institute in Dakar, where the mouse-adapted
French strain of yellow fever virus was used for the production of the
“Dakar vaccine”. This vaccine has been used on a mass scale in the
French-speaking areas of Africa, where it was found to be adequately
stable and highly effective when administered, usually in combination
with smallpox vaccine, by scarification of the skin. In some countries,
however, cases of encephalitis occurred after its use, and in several
instances it was proved that these were related to infection with the
vaccine.

Max Theiler and co-workers at the Rockefeller Foundation in New
York undertook to develop a strain attenuated for both neurotropism
and viscerotropism by serial propagation of the virus in various types
of tissue culture. The 17D virus line grown in tissue cultures of chick
embryos, from which the central nervous system tissue had been
removed, and passed 114 times satisfied their criterion of showing
negligible disease in intracerebrally inoculated rhesus monkeys. It was
shown to produce a safe and effective vaccine for human use at pas-
sage 204.

Since 1937 the 17D vaccine produced in chick embryos has been
used on an increasing scale, first in Latin America, and then through-
out the world. Initially, multiple passages of 17D virus were mainte-
nined in tissue culture in different laboratories and vaccine lots were
produced as needed from the current passage level of the available
subline. By 1943, however, three significant problems had been en-
countered in 17D vaccination programmes sponsored by the Rocke-
feller Foundation.
First, some failures of the vaccine to immunize were observed; these were associated with vaccine lots produced from virus that had been subcultured many times (350 passages). Such virus, as was shown later, had lost much of its infectivity for man, although it still produced lethal encephalitis in mice inoculated by the intracerebral route. This problem was resolved by reverting to an earlier passage of virus for vaccine production.

Secondly, a few cases of vaccine-associated encephalitis were observed in Brazilian children. A review of tests of lots of vaccine produced from the several passage sublines being maintained in parallel (see Chart 1, p. 40) revealed that the incidence of encephalitis in the test monkeys differed appreciably according to the sublines from which the vaccine lots were derived (3).

The significance of this finding was confirmed in large-scale field studies which included a carefully controlled trial of vaccines produced from each of five 17D substrains (4, p. 117). The problem, as well as that of diminishing immunogenicity, was resolved by selecting, for the production of a master seed lot, the safest substrain at the passage level at which it was used in the field trial. Secondary seed lots that were used for production lots were prepared from this master seed lot, thereby ensuring that vaccine was produced within a narrow range of passages.

The third problem was the occurrence of serum hepatitis in recipients of vaccine. Although smaller episodes associated with yellow fever vaccine had been noted previously in both England and Brazil, the first major occurrence of vaccine-associated hepatitis was recognized in Brazilian populations in 1941 (4, p. 68) and was followed by a much larger one in United States military personnel (5, 6). The cause was “normal” human serum (heated at 56°C) incorporated into the vaccine as a source of “inocuous” protein to protect the virus during lyophilization and to facilitate subsequent rehydration of the vaccine as well as to stabilize the vaccine virus once reconstituted. The solution was to omit the serum, and to increase the concentration of embryo tissue fluid in the final product, thereby providing a protein concentration adequate for lyophilization and virus stability.
2. PROCEDURE FOLLOWED SINCE 1952 FOR APPROVAL BY WHO OF LABORATORIES FOR YELLOW FEVER VACCINE PRODUCTION

2.1 Background

When the Fourth World Health Assembly on 25 May 1951 adopted the text of the International Sanitary Regulations, it retained with slight modifications, a provision which appeared in Article 1, paragraph X of the International Sanitary Convention for Aerial Navigation of 12 April 1933, as amended by the International Sanitary Convention for Aerial Navigation, 1944. The part of this Convention to which reference is made reads as follows:

"Article 1 (Article III of the Amendment)
...
"X. A valid anti-yellow fever inoculation certificate is one certifying that the bearer has been inoculated against yellow fever, with a vaccine and by a method approved by UNRRA ."

In addition, the International Sanitary Convention for Aerial Navigation, 1933/1944, specifies:

"Article 36 (Article XI of the Amendment)
...
"(10) That UNRRA shall lay down standards with which yellow fever vaccine shall conform.
"(11) That they will make arrangements to test at frequent intervals the activity of the yellow fever immunizing vaccine in use in order to ensure that its immunizing properties are satisfactory, and for this purpose agree that UNRRA in consultation with the governments concerned and, as regards the Western Hemisphere, with the Pan American Sanitary Bureau, shall designate from time to time institutes which are approved for the carrying out of such tests."

At its fourth session, in 1947, the WHO Interim Commission decided to have tests of vaccine activity carried out by two or more approved control laboratories on all yellow fever vaccine for which its approval was asked in accordance with the provisions of Article 36 (11) of the 1933/1944 International Sanitary Convention for Aerial Navigation. That procedure has been retained by WHO and has been followed since the entry into force of the International Sanitary Regu-

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lations on 1 October 1952 on each occasion on which the Organization’s approval has been requested.

2.2 Procedure

WHO considers only applications for its approval transmitted through the health administration of the country in which the institute or laboratory preparing the yellow fever vaccine is situated. Applications are to be accompanied by a report giving detailed information with regard to:

(a) the personnel responsible for the preparation of the vaccine (such personnel must not engage in any work on other viruses or bacteria for the period of the preparation of the vaccine);
(b) the premises and material used (which must not be used for any other purpose);
(c) the preparation technique (inoculation and incubation of eggs, harvesting and grinding of embryos, filling of ampoules and desiccation must all be carried out in conformity with the approved standards);
(d) the control tests (which must give satisfactory results).

The activity of the vaccine is then tested. This testing is done by three laboratories selected from the list of institutes and laboratories approved for the purpose by WHO.

The required number of ampoules (generally six) of a single batch of vaccine, packed in dry-ice, are sent by air to the three control laboratories. A copy of the protocol established by the institute manufacturing the vaccine, giving the details of the preparation and the results of the titration, are sent direct to the World Health Organization but not to the control laboratories, which titrate the vaccine independently and communicate the results to WHO.

The results of the titrations made by the control laboratories and by the institute which prepared the vaccine are then compared and communicated to certain members of the WHO Expert Advisory Panel on Yellow Fever together with a summary of the report accompanying the application for approval. The experts are requested to give an opinion as to whether WHO can accord its approval to the vaccine tested. They may call for further information and, if they deem it necessary, a second control test.

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The replies of the experts are centralized by the secretary to the Expert Advisory Panel on Yellow Fever, and until 1953 they were submitted to the Executive Board of WHO. If they were all favourable the Board adopted a resolution approving the yellow fever vaccine prepared by the institute or laboratory for the issue of international vaccination certificates.

Since 1954, the Director-General has been authorized by the Executive Board "subject in each case to the satisfactory completion of the technical procedure now established, to grant approval to yellow fever vaccines for the issue of the international certificates of vaccination and revaccination".

The decision is communicated to the health administration concerned and published in the WHO Weekly Epidemiological Record.

3. RELEVANCE OF PRESENT REQUIREMENTS

3.1 General Requirements for Manufacturing Establishments and Control Laboratories

The General Requirements for Manufacturing Establishments and Control Laboratories (7) shall apply to the manufacture and control of yellow fever vaccine. In particular it is necessary to ensure that, during production, the vaccine does not become contaminated with any other bacteria or virus from the surroundings. This demands isolation of the premises, both physically and by means of separate supplies of clean air.

In some countries the sequential production of different vaccines in the same facility is permitted. In such circumstances the production facility must be sterilized before the production of yellow fever vaccine begins.

3.2 Requirements for Yellow Fever Vaccine

In a series of meetings of the WHO Expert Committee on Biological Standardization, various recommendations have been made for improving the quality of the vaccine and for surveillance of its production. Finally revised Requirements for Yellow Fever Vaccine were accepted by the Expert Committee in 1975 and published in 1976 (2, pp. 23–49).
Chart 1. History of the seed virus used for the preparation of yellow fever vaccine

December 1933. Tissue cultures initiated with the virus from the serum of a monkey infected with the unmodified Asibi strain of yellow fever virus.

18th
- 18 passages in cultures containing mouse embryo tissues, Tyrode solution, and normal monkey serum.

68th
- 50 passages in cultures containing whole chick embryo tissues, Tyrode solution, and normal monkey serum.

204th
- 138 passages in cultures containing whole chick embryo tissues from which head and spinal cord had been removed, Tyrode solution, and normal monkey or human serum (not inactivated).

221st
- 17 passages in cultures containing chick embryo tissues from which head and spinal cord had been removed, Tyrode solution, and normal human serum (not inactivated).

225th
- 1 chick embryo passage for preparation of vaccine lot No. N.Y.75.
- 4 passages in cultures containing chick embryo tissues from which head and spinal cord had been removed, Tyrode solution, and human serum.
- 1 passage in chick embryo for preparation of Colombia vaccine No. 70.
- 1 passage in chick embryo for preparation of Colombia vaccine Nos. 88 and 90.
- 1 passage in chick embryo for preparation of seed virus for vaccine production in New York.

3.2.1 Seed lots at present in use

In the past several substrains were derived from the original 17D strain, as shown in Charts 1 and 2. Most of the seed lots at present utilized in laboratories producing yellow fever vaccine originated from substrain 17D-204, and some originated from substrain 17D low. After consultation with the vaccine producers, the genealogy of the seed lots which they use has been traced; it is shown in Charts 3 and 4.

Primary seed lots derived from substrain 17D-204 (Chart 3) represent the 231st to the 236th passage of the virus, and the vaccine represents two additional passages. This is one passage more than is

**Chart 2. Derivation of substrains of 17D virus used in Brazil**

- Subculture
- Passages made in: NEW YORK, BRAZIL
- Substrain designation: Passed in tissue culture, Passed in chick embryo

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*Source: Fox, J. P. & Penna, H. A. American journal of hygiene, 38: 152 (1943).*
indicated by most of the producers of vaccine. The Bureau of Biologics, Food and Drug Administration, Bethesda, MD, USA, has indicated that Colombia 88 seed was the 228th passage because the first chick embryo passage to prepare the vaccine lot NY 78 has been omitted on the count formerly established by Dr. M. Theiler (see Chart 1) after the 221st passage.

Primary seed lots derived from substrain 17DD low represent the 284th and 285th passage of the virus in this series (Chart 4).

3.2.2 Necessity for a leucosis-free primary seed lot

During the last five years several manufacturers have freed their yellow fever virus seed from the avian leucosis viruses and are now producing vaccine free from detectable living contaminants. The passage level of seed lots that have been freed from leucosis viruses are indicated in Chart 3, in parallel with the seed from which they were derived.

A requirement of some national control authorities is that eggs used for the production of vaccine should be shown by suitable tests to be free from avian leucosis virus (ALV), mycoplasma, and other agents: this recommendation requires further consideration in the light of the recent development of 17D virus seed lots free from ALV. The testing of the seed virus for neurotropism in susceptible monkeys remains a mandatory requirement and also requires further considera-
tion in the light of possible changes in the virus brought about by the manipulations to eliminate ALV.

When WHO reformulated the Requirements for Yellow Fever Vaccine in 1975 (2), several changes were made in order to incorporate advances in technical methods, particularly with respect to the titration of virus content of the vaccine. It was not possible, however, to demand that all vaccine should be free from avian leucosis viruses.

3.2.3 Provision of a primary leucosis-free seed lot by WHO

In the WHO Requirements for Yellow Fever Vaccine formulated in 1958 manufacturers were required to use a primary or secondary seed lot of the 17D virus strain that had been fully tested (1). A suitable strain could be obtained by application, through WHO or directly, to a specialized laboratory. Several applications have been received since that time but all materials available, though otherwise suitable, have been contaminated with avian leucosis viruses. However, there was no single virus preparation that was recognized as the WHO seed.

In future, it will be necessary to have available a source of suitable yellow fever virus free from contaminants, and WHO has taken steps for an officially designated seed satisfying this requirement to be provided on request.

WHO has made arrangements with the Robert Koch Institute, Berlin (West), for the provision of a WHO leucosis-free primary seed lot. The primary seed derived from the substrain 17D-204 at the 236th passage and designated 213-77 is in the process of being tested for freedom from extraneous agents and neurotropism. The vaccine produced from the WHO primary seed through the intermediary of a working secondary seed will be at the 239th passage level. This is a later passage level than that of any vaccine in use today, and careful surveillance of subjects given vaccine at this level is important.

3.2.4 WHO's reference preparation for potency testing

The potency of yellow fever vaccine is measured by its virus content. The more modern technique of measuring the virus content in cell cultures has, in most laboratories, replaced that of measuring infectivity in mice. An international collaborative study showed that virus plaque assays in Vero or PS cells, using a microtitre technique, gave more reproducible results than assays in mouse brains. Furthermore,
the inclusion in the titration of a reference preparation as a control of the sensitivity of the cell system and a subsequent adjustment to a standard titre gave greater uniformity to the virus titres obtained (8). Accordingly a WHO reference preparation for use in the determination of potency has been prepared and the collaborative study has shown it to be suitable; it is now available to national control authorities for the calibration of national standards.3

3.2.5 International Reference Preparation of Anti-Yellow Fever Serum

The International Reference Preparation of Anti-Yellow Fever Serum is distributed for the establishment of national reference sera to be used: (i) in the test for identity, and (ii) in the measurement of antibody responses to yellow fever vaccine.

In its use in the identity test it is important to ensure that the serum, in the dilution in which it is used, does not neutralize other flaviviruses (group B arboviruses). This would mean that only yellow fever virus would be neutralized, and the test would thus also serve as an additional test for the presence of extraneous agents.

4. RECOMMENDED REVISED PROCEDURE FOR APPROVAL BY WHO OF YELLOW FEVER VACCINE

4.1 Facilities, staff, and operating procedures

The General Requirements for Manufacturing Establishments and Control Laboratories (7) adequately cover the quality of the buildings and equipment required for vaccine production. In the case of yellow fever vaccine, however, WHO requires additional information as follows:

(1) A plan of the production facility shall be submitted to WHO for approval, as well as information on all activities in adjacent buildings. An assurance of the isolation of the facility is necessary, particularly with respect to entry by unauthorized personnel and the existence of a separate supply of clean air.

3Available from: Chief, Biologicals, World Health Organization, Geneva, Switzerland.
(2) The methods used to produce and test the vaccine, as outlined in the standard operating procedures of the facility, shall be registered with WHO. Any methods or tests that differ from the WHO Requirements shall be mentioned specifically.

(3) The names and qualifications of the senior staff involved in vaccine production and testing, and particularly those who will be signing any part of the protocol, shall be registered at WHO. Such staff shall have had a training in the basic sciences with experience in microbiological techniques, particularly those involved in the production and testing of virus vaccines.

4.2 Testing of primary and secondary seed lots

When the manufacture of yellow fever vaccine is being undertaken for the first time, a primary seed that has been fully tested must be used. The results of the tests must be available to the manufacturer and WHO.

The secondary (working) seed prepared by the manufacturer must undergo full testing in two referral laboratories approved by WHO in addition to that of the manufacturer. The protocols reporting the results of all tests are then submitted to WHO and the referral laboratories and a decision is made as to the suitability of the secondary seed.

4.3 Establishment and maintenance of consistency

One of the most important criteria in the safety of a live virus vaccine is consistency of production.

Although the need to establish such consistency has been recognized, the criterion has not been applied in the last 20 years. The Working Group felt that, for new manufacturers of the vaccine or for vaccines produced from a new secondary seed by a WHO-approved manufacturer, a consistency record of production should be established by the examination of the first five consecutive lots of vaccine prepared from the secondary seed for:

(i) freedom from extraneous agents (except, in some cases, for avian leucosis viruses);
(ii) virus titre of vaccine before and after freeze-drying;
(iii) stability by maintenance of virus titre when held for two weeks at 22 °C.
The results of tests of each consecutive lot should be submitted to WHO to demonstrate that consistency has been established.

The maintenance of consistency will be shown by the submission of protocols of production and testing for each batch of vaccine prepared; these will be used for international certification of vaccination. In addition, breaks in consistency should be brought to the attention of WHO.

4.4 Visits to manufacturing establishments

Past experience has shown that much can be gained by discussions of production methods and test procedures with the staff, particularly when the manufacturer is new. This approach also offers an opportunity to consider the most suitable design for the facility. The discussions can take place with the national control authority, but WHO staff and experts are available to take part in them, on request. In any event there should be a report to WHO indicating that the facility is suitable for vaccine production.

4.5 Approval of production laboratories

When, starting in 1948, WHO was initially involved in the approval of vaccine manufacturing facilities, the results of the relevant tests, together with an overall report, were presented to the WHO Executive Board and subsequently considered for approval by the World Health Assembly. In 1954, by resolution EB13.R52, the Board authorized the Director-General to approve new manufacturers for the production of yellow fever vaccine used in connexion with international certification. The system of presenting a report of experts on the subject to the Director-General for approval seems to be the most appropriate one and no changes are suggested.

4.6 Modifications in vaccine production necessitating a reassessment

Past experience has shown that 17D yellow fever vaccine is safe as long as it is prepared by standardized methods, as described in the Requirements for Yellow Fever Vaccine (2). It is possible that changes introduced into the procedure may induce a phenotypic selection of virions giving rise to undesirable variations in the properties of the vaccine. Such changes include the use of certain techniques for
the removal of avian leucosis viruses, for example: (i) passage at high
dilution, (ii) filtration, (iii) selection by growth at different tempera-
tures, (iv) differential centrifugation, or (v) growth of the virus in
the presence of an antiserum to the leucosis viruses. Propagation of
the virus in tissues including cell cultures other than the chick embryo
may also give rise to phenotypic variations.

If any laboratory approved by WHO for the production of yellow
fever vaccine wishes to introduce such changes in the standard operat-
ing procedures for production, it must first notify WHO, which will, if
necessary, submit the proposals to an independent panel of experts,
who may suggest further safety testing.

4.7 Approved laboratories

For many years, a number of laboratories have been approved by
WHO for the production of yellow fever vaccine; a list of these is
given in the Appendix. Each laboratory has been approved after
submission of the necessary vaccines and data. A new secondary seed
produced by these laboratories shall, however, pass the test procedure
previously outlined before the batches of vaccine produced from the
new seed are approved.

4.8 Arrangements for countries importing vaccine

For those countries in which yellow fever vaccine is not produced,
the WHO Requirements for Yellow Fever Vaccine (2, p. 45) include
a summary protocol for the purpose of reporting the results of tests
applied during production and quality control. In this way, compliance
with the WHO Requirements can be confirmed.

If a country does not have any staff available for reading such
protocols, WHO is willing to lend assistance in this respect. It is
important to bear in mind that approval can be given only on a batch-
by-batch basis, and that a manufacturer cannot be given blanket
approval for all batches.

\* Requests for such assistance should be made to Chief, Biologicals, World Health
Organization, Geneva, Switzerland.
5. PROBLEMS RELATED TO THE USE OF VACCINE

5.1 Duration of immunity after yellow fever vaccination

The validity of the international certificate was extended from six to ten years by the Eighteenth World Health Assembly in 1965 (resolution WHA18.5). The persistence of neutralizing antibodies up to 17–19 years has been described (9, 10), and it has been assumed that protection may persist for longer. In 1978 a study was undertaken jointly by the Yale Arbovirus Research Unit, Yale University, New Haven, CT, USA, and the Center for Disease Control, Atlanta, GA, USA, on 125 sera of veterans of the Second World War vaccinated in 1943. It was found that 62% of the sera conferred protection or partial protection in a test in six-week-old mice inoculated intracerebrally with 31–630 mouse LD₅₀ of French neurotropic virus, and the results correlated well with a plaque reduction neutralization test in Vero cells. Two difficulties were encountered during a study of the duration of antibodies, however: (i) the protection may persist longer than is shown by circulating antibodies, and (ii) vaccinated individuals may have had a boost in antibody titre resulting from post-vaccination contact with other flaviviruses.

The Group was of the opinion that there was not enough evidence to justify an extension of the period of validity of the certificate beyond 10 years.

5.2 Yellow fever vaccination during pregnancy

In 1979 a survey was carried out by WHO in selected vaccinating centres and health services to determine the risk of fetal damage when 17D yellow fever vaccination was given during pregnancy. While the relevant data were not methodically recorded in most centres, the information collected did not provide any evidence that an exceptionally high risk of such damage was associated with vaccination during pregnancy. Nevertheless, the Group felt that it was better to play safe and not to vaccinate during pregnancy, particularly during the first three months. The hypothetical risk associated with vaccination, however, has to be weighed against the risk of natural infection, the latter being certainly the greater of the two during epidemics and for persons living in yellow fever endemic areas.
6. CONSTRAINTS AND IMPROVEMENTS

6.1 Shortage of monkeys

The WHO Requirements for Yellow Fever Vaccine make it mandatory to carry out tests in monkeys to establish the freedom from neurotropism of the virus seed used for vaccine production (2, p. 35). For the last 20 years all producers have used *Macaca mulatta* for this purpose, because until recently this species, known to be susceptible to the yellow fever virus, was readily available. Now monkeys of this species are no longer available from the two countries from which they were obtained, and there is an urgent need to breed them in captivity or to investigate whether other species of monkeys are equally susceptible.

The Group suggested that strains of yellow fever virus known to differ in their reactivity in man and in rhesus monkeys should be located and tested in several species of monkeys. Furthermore, such strains could be used in a search for markers that could be correlated with monkey neurovirulence.

6.2 Desirability of further research on scratch vaccines

Yellow fever has recently been active in West Africa and the Caribbean, necessitating extensive mass vaccination of the human population with 17D vaccine given subcutaneously. It is probable that this need will arise again in these areas and elsewhere.

Mass vaccination could be carried out more quickly and cheaply if it were done by skin scarification rather than by subcutaneous injection. The French neurotropic strain, prepared in mouse brain, has been used widely for vaccination by scarification, but has caused encephalitis, particularly in children. Over 25 years ago, it was shown that the 17D strain could be administered successfully by skin scarification. The first trials were done with 17D strain grown in chick embryos, but later trials showed that better results were obtained with the same strain grown in a single passage of mouse brain, in which high titres of virus can be obtained. However, by present-day standards, the latter tissue is unlikely to be accepted as suitable for the preparation of vaccine for routine use in humans although it may sometimes have to be considered in an emergency.

Little further experience on vaccination by scarification has been gained during the last 15 years. It is recommended that more trials
should be done, using the 17D strain grown in chick embryos and administered by the bifurcated needle, which was so successful in the smallpox eradication campaign. Graded doses of vaccine should be used, and the effect of pre-existing antibodies to other flaviviruses should be studied.

6.3 The stability of vaccines

Only vaccines known to be relatively thermostable should be used in the countries with high ambient temperatures where yellow fever vaccine is largely used.

Most vaccines can be kept at +4°C for at least one year, which means that in a continuously operating domestic refrigerator there is no need for −20°C storage. Many vaccines will maintain adequate virus titre (≥ 10^9 mouse LD50 per human dose) for two weeks when held at 22°C and some even have a half life of ten days at 37°C. In all countries with warm climates, however, the vaccine should be stored at +4°C to −20°C.

As suggested by the WHO Requirements a vaccine that is stable when held for two weeks at 37°C is required (2, p. 42).

The Group recommended that data on the stability of yellow fever vaccines in hot climates should be assembled and the value of stabilizers should be assessed.

7. RECOMMENDATIONS

The aim is to have a freely available, stable, and nonreactive vaccine that can be given by a simplified technique and confer life-long immunity on more than 95% of vaccinees.

The Group considered it essential that WHO should continue surveillance over the production of yellow fever vaccine for the following reasons:

(1) to maintain the standards of potency and safety of this non-stable live product used to ensure the continuing protection of countries at present free from yellow fever but recognized as receptive to yellow fever;

(2) to reassure the authorities of countries having to import vaccine that it conforms to internationally acceptable standards; and

(3) to ensure the good quality of vaccine made by new producers.
Having examined recent progress in the preparation of yellow fever vaccine, the Working Group recommended that the procedures indicated in section 4 of this Annex should be followed for obtaining approval by WHO of laboratories producing a vaccine for international vaccination certification.

The need was emphasized for further research on how to simplify the administration of yellow fever vaccine by the percutaneous route and increase the heat stability of vaccines.

**COMPOSITION OF THE WORKING GROUP**

The Working Group on the Procedure for Approval by WHO of Yellow Fever Vaccines consisted of the following WHO advisers and staff members:

Dr P. Brès, Virus Diseases, World Health Organization, Geneva, Switzerland

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Dr W. C. Cockburn, Lothian Board of Health, Edinburgh, Scotland (*Adviser*)

Dr C. C. Draper, London School of Hygiene and Tropical Medicine, London, England (*Adviser*)

Professor J. P. Fox, School of Public Health and Community Medicine, University of Washington, Seattle, WA, USA (*Adviser*)

Dr J. H. S. Gear, National Institute of Virology, Sandringham, Transvaal, South Africa (*Adviser*)

Dr F. T. Perkins, Biologicals, World Health Organization, Geneva, Switzerland

**REFERENCES**


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Appendix

LABORATORIES APPROVED BY WHO FOR THE PRODUCTION OF YELLOW FEVER VACCINE

Commonwealth Serum Laboratories
Parkville, Victoria
Australia

Oswaldo Cruz Institute
Rio de Janeiro
Brazil

National Institute of Health
Bogotá, D. E.
Colombia

The Wellcome Research Laboratories
Beckenham, Kent
England

Pasteur Institute
Paris
France

Robert Koch Institute
Berlin (West)

Central Research Institute
Kasauli, Himachal Pradesh
India

Royal Tropical Institute
Amsterdam
Netherlands

Federal Laboratory Service
Lagos
Nigeria

Pasteur Institute of Dakar
Dakar
Senegal

National Institute for Virology
Sandringham, Transvaal
South Africa

Connaught Laboratories Inc.
Swiftwater, PA
USA
Annex 2

REQUIREMENTS FOR RABIES VACCINE
FOR HUMAN USE

(Requirements for Biological Substances No. 22)
(Revised 1980)

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INTRODUCTION

Although the first set of Requirements for Rabies Vaccine for Human Use (1) were formulated less than 10 years ago (1973), the technical developments that have taken place since then justify their
revision. Accordingly, the WHO Expert Committee on Biological Standardization, in its twenty-ninth report (2), requested the WHO Secretariat to arrange for such revision.

The following international requirements for rabies vaccine (for human use) have been fitted into the framework adopted in the Requirements for Biological Substances Nos. 1 to 28, already published by WHO (2, p. 142), and, in drafting them, account has been taken of the opinions of consultants, the regulations and requirements for the manufacture and control of rabies vaccine that have been formulated in several countries, as well as information from both published and unpublished reports. In addition, comments have been received from a number of experts (see Acknowledgements, page 87).

GENERAL CONSIDERATIONS

Rabies vaccines at present used in man in most countries are derived from animal neural tissue, but in some countries the vaccines in most common use or under development are those prepared in duck embryos or in cell cultures. These are referred to in the report of a meeting held in Marburg in 1977 (3). Because of the widespread manufacture and use of rabies vaccine from neural tissue and embryos and the rapid development and use of cell culture vaccines at this time, these revised international requirements for rabies vaccine have been written to cover all types of vaccine. Furthermore, in view of the recommendation of the WHO Expert Committee on Rabies, in its sixth report (4, p. 17), the requirements have been restricted to inactivated vaccines.

A number of different manufacturing and testing procedures are in use in various countries. The procedures differ in the rabies virus strain used (there are now three in common use), the species of animal used for propagation of the virus, the types of cell cultures used, the method of inactivation, the preservatives added, the form in which the vaccine is used, and the methods for testing potency.

The passage histories of the different strains of virus being used for production are not well documented. Such a strain should be one known to produce vaccine that is antigenically active against classical rabies virus strains (i.e., those belonging to serotype 1 of the rabies subgroup of rhabdoviruses). While many strains have been derived from the original Pasteur strain, others used for production have been isolated more recently from man or animals. Strains used for produc-
tion should be limited to what is termed a “fixed” strain of virus. This is one that has a short, stable, and reproducible incubation period when injected intracerebrally into suitable animals. However, it can be demonstrated that strains in use at the present time differ considerably in their ability to produce rabies in experimental animals when inoculated by a route other than the intracerebral. The present requirements recommend that the Pasteur strain of rabies “fixed” virus maintained originally only in rabbits by intracerebral inoculation should be used for production. The method of maintaining the “fixed” virus today, however, does not involve only rabbit inoculation. The use of the seed lot system has also been specified, and since the International Standard for Anti-Rabies Serum is not made widely available it is recommended that a specific anti-rabies serum should be produced and made available by national control authorities as an aid to establishing the identity and purity of the seed virus. Although in the first requirements it was suggested that studies should be made to establish appropriate genetic markers to characterize the virus used for production, none has been found, except that “fixed” virus does not form Negri bodies. It was assumed that the ability of a rabies vaccine to produce high neutralizing antibody titres in man is an indication of its effectiveness in protection, and that good protection in experimental animals is an indication that a vaccine will be effective in man. There is now much more evidence indicating that vaccines giving high antibody levels in man or animals will protect against rabies. In several studies in which human diploid cell culture vaccines of high potency (2.5 IU) have been given to persons bitten by rabid animals within a period not exceeding 72 hours after the event, none of the subjects has died from rabies. However, more experience with persons severely bitten by rabid animals is necessary in order to assess the full effectiveness of human diploid cell culture vaccines. Although it may not be possible to specify the minimum level of activity that will afford protection for man, it is now known that vaccines with a potency of 2.5 IU per dose in a six-dose schedule given after exposure have prevented the development of rabies in man. It is important that rabies vaccines should be prepared that have maximum antigenicity in man and experimental animals, together with an acceptably low level of adverse reactions. For this reason it is advisable that a preparation of rabies vaccine should be diluted to the concentration at which it just satisfies minimum potency requirements.

Neural tissue rabies vaccines have been in worldwide use for generations, and experience has indicated that they are normally effective.
It is generally accepted that the risk of central nervous system involvement in recipients of vaccine prepared in the brains of adult animals is about 1 in 2000 doses administered. Such adverse reactions are greatly reduced when the virus is grown in the brains of newborn animals, such as rats and mice, before the development of myelin in the brain, and the risk associated with duck embryo vaccine is about 1 in 11,000 doses administered. However, unless it is concentrated by some means, duck embryo vaccine is somewhat less immunogenic in mouse potency tests than is neural tissue vaccine, and it does not consistently produce a good level of neutralizing antibody in man. Nevertheless, statistical evidence has shown that the development of rabies following an animal bite is not more frequent in subjects given duck embryo vaccine than in those given neural tissue vaccine. Because of its greater safety, duck embryo vaccine is being increasingly used for pre-exposure immunization. Studies have been made of the effectiveness of duck embryo vaccine in combination with the recommended amounts of antirabies human immunoglobulin (20 IU per kg of body weight) for post-exposure treatment. If heterologous immunoglobulin is used, 40 IU per kg of body weight should be given.

Because of the nature of the disease, it is virtually impossible to do controlled clinical studies involving an unvaccinated group to determine accurately the degree of effectiveness of the rabies vaccine. The development of highly potent and safe vaccines, however, has allowed their use prophylactically, and it is now known that the tissue culture vaccines give high antibody levels in nonimmune subjects.

It has been shown in various post-exposure animal models that the combination of interferon and rabies vaccine or interferon inducers and vaccine is more effective than vaccine alone. These regimes appear to be effective because of the local production or introduction of interferon; at all events, the role of interferon in providing protection in man is currently being investigated.

The potency testing of rabies vaccine is of considerable concern. The potency test must be capable of discriminating between vaccines of different activity, and preferably a single test should be applicable to all types of vaccine. The rabies virus strain used for challenge should be one of reproducible virulence for the test animal when given intracerebrally. In addition, the test should be reproducible and economical. A common reference preparation of vaccine is of importance in evaluating test results. Ideally, such a reference preparation should be protective in animals and shown to produce antibodies in man. The third International Reference Preparation of Rabies Vaccine, estab-
lished in 1978 (5, p. 15), has been tested in man and shown to protect and give an antibody response. Classically, for neural vaccines, potency has been based on the wet weight (mg) of neural tissue required for the protection of 50% of the test animals. However, in the case of purified vaccines that contain reduced amounts of host tissue, as well as in that of cell culture vaccines, the potency should be expressed on the basis of the dilution of vaccine (injected in a defined volume) protecting 50% of the test animals rather than on the basis of the tissue content.

The International Reference Preparation of Rabies Vaccine, when reconstituted from the dry form according to instructions, has an activity of 10 IU per ampoule. For determining the potency of any vaccine in animals, serial dilution is made, starting from the concentration at which the vaccine is administered to man, or the equivalent concentration of the reference vaccine, as the case may be. By this procedure a single reference vaccine may be used for routine potency testing of all types of rabies vaccine. This has the additional advantage of providing a common basis for comparing the potency of the classical neural tissue vaccines, with which there have been many years of experience, and the potency of the newer types of vaccine.

It is important also to consider the inclusion of an antibody response test in animals, since this has been shown to be similar to that given in man and to discriminate between vaccines of different potencies.

Tests for factors in the vaccine that may induce allergic encephalomyelitis have not been included in these requirements, because no techniques have been described that can be relied on. The degree of reproducibility of existing procedures has not been evaluated and there is evidence that considerable variation in results can be expected. Existing tests, however, can be used to assess the period during which the factor causing allergic encephalomyelitis develops in the brain of young animals. Studies should continue to be encouraged for improving such tests as long as neural tissue vaccine is used.

Because of the need for large quantities of vaccine in areas in which rabies is prevalent, in some countries vaccine is used without fulfilling all the requirements for the potency test, in order to avoid serious curtailment of vaccine production. This, however, is not a satisfactory situation and all the present requirements should be complied with.

The use of healthy animals has been specified in these requirements, but no provisions are incorporated for tests for adventitious agents. National control authorities should, however, pay attention
to the problems of ensuring that the animals used are free from infectious agents that might contaminate rabies vaccine. National authorities should also discourage the use of the brains of adult animals for the production of vaccine. A determined effort should be made to avoid this practice, either by the use of immature animals (in which neuroallergic activity has not yet become demonstrable), such as suckling mice, or by the use of cell cultures.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements, so that, if a health administration so desires, these parts as they appear may be included in 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 rabies vaccine, it is recommended that a clause should be included permitting modifications of manufacturing requirements on the condition that it can 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 and/or used.

PART A.
MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be “Vaccinum rabiei (ad usum humanum)”. 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.

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1.2 Descriptive definition

Vaccinum rabiei (ad usum humanum) is a fluid or dried preparation of rabies “fixed” virus grown in the neural tissues of rabbits, sheep, goats, mice, or rats, or in embryonated duck eggs, or in cell cultures, and inactivated by a suitable method. The preparations for human use shall satisfy all the requirements formulated below.

In view of the increased stability of the dried vaccine its use should be encouraged.

1.3 International Reference Preparation and International Standard

The International Reference Preparation of Rabies Vaccine, established in 1978 (5, p. 15), is stored and distributed in ampoules containing freeze-dried rabies vaccine, prepared in human diploid cells and inactivated with beta-propiolactone. There is an activity of 10 IU per ampoule. This reference preparation is intended for the calibration of national reference preparations for use in tests of potency of rabies vaccine (see Part B, section 1).¹ After reconstitution, the International Reference Preparation may be stored in suitable aliquots for subsequent animal immunization, provided that the storage temperature is below −60°C and that the period of storage is not longer than one month.

The International Standard for Anti-Rabies Serum, established in 1955 (6, p. 11), is stored and distributed in ampoules containing 86.6 IU. This standard is intended for use in the laboratory assay of potency of antirabies immunoglobulin preparations used in man. It can also be used for the assay of rabies antibodies in man and animals.

The reference preparation and the standard 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.

1.4 Terminology

Master seed lot: a quantity of virus that has been processed together and has a uniform composition. It is used for vaccine preparation or for the preparation of further working seed lots.

Working seed lot: a working seed lot which is prepared from the master seed lot and which shall be not more than 5 passages removed from it.

¹ Use of the International Reference Preparation for administration to man is not authorized. A national reference preparation should not be considered as suitable for use in man unless it has been approved by the national control authority.
Cell seed: a quantity of cells derived from a single human or animal tissue and of uniform composition, stored frozen at \(-70^\circ\text{C}\) or below in aliquots, one of which would be used for the production of a single harvest.

Single viral harvest: virus harvested from a single animal or from a group of suckling animals or a group of embryonated eggs or cell cultures inoculated at the same time and harvested together. The virus harvested from cell cultures may be taken on more than one occasion and subsequently pooled. The virus in the harvest is without intervening passage from the working seed lot.

Bulk material: a pool of inactivated single harvests before preparation of the final bulk. It may be prepared from one or a number of single harvests and may yield one or more final bulks.

Final bulk: the finished biological preparation present in the container from which the final containers are filled. It may be prepared from one clarified bulk suspension or from a blend of clarified bulk suspensions.

Filling lot (final lot): a collection of sealed final containers, dispensed from the same final bulk, that are homogeneous with respect to the risk of contamination during filling or drying. A filling lot must therefore have been filled in one working session and (if applicable) have been dried together in the same chamber.

Tissue culture infective dose 50% (TCID\textsubscript{50}): the quantity of a virus suspension that will infect 50% of cell cultures.

Mouse lethal dose 50% (mouse LD\textsubscript{50}): the quantity of a virus suspension that will kill 50% of mice injected with it.

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) (7, p. 11) shall apply to establishments manufacturing rabies vaccine for human use.

The production of rabies vaccine shall be conducted by staff who have not handled other infectious microorganisms or animals in the same working day. The staff shall consist of persons who shall be examined medically and found to be healthy. Steps shall be taken to ensure that all such persons in the production and control areas have
been immunized against rabies and have an antibody titre of at least 0.5 IU per ml of serum.

Only the cultures approved by the national control authority for the production of rabies vaccine shall be introduced or handled in the production area.

Persons not directly concerned with the production processes, other than official representatives of the national control authority, shall not be permitted to enter the production area.

Particular attention shall be given to the recommendations contained in Part A, section 1, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) 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 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) (7, p. 15) shall apply to the manufacture of rabies vaccine.

3.1 Control of source materials

3.1.1 Strain of virus

The strain of virus used in the production of all seed lots shall be a "fixed" strain and shall be identified by historical records. It shall have been shown, to the satisfaction of the national control authority, to yield safe and immunogenic vaccines when the virus has been inactivated. In addition, the vaccine strain shall be characterized by serological tests and animal inoculation.

Records shall be maintained of all tests for verification of strain characters to be made each time the working seed is changed. Such tests shall include the titration in animals of various species and ages and by various routes of inoculation as well as serum neutralization
tests. The tests shall be those approved by the national control authority.

The most common production strains originate from the Pasteur strain of rabies "fixed" virus, maintained historically in rabbit passage, or a derivative of such a strain.\textsuperscript{2} Such strains should be capable of producing characteristic paralysis within 5–7 days when inoculated intracerebrally into animals.

3.1.2 Substrates for the production of seed virus and vaccine

3.1.2.1 Animals. Only healthy animals shall be used. They shall conform to the requirements given in Part A, section 3.2.1.

Different species of animals may be used for vaccine production or for preparing seed virus. Rabbits (adult or preferably suckling), sheep, goats, suckling mice, and suckling rats are used in different countries.

3.1.2.2 Embryonated eggs. Only embryonated eggs obtained from healthy flocks shall be used. The birds shall be routinely monitored to ensure the absence of Salmonella infections, Mycobacterium avium, and other pathogenic microorganisms and viral agents naturally occurring in ducks.

In some countries the whole group of birds is bled on the establishment of the flock, and thereafter a 5% sample of the birds is bled each month. The serum samples are screened for freedom from antibodies to the pathogens for the particular ducks. Any bird that dies is investigated to determine the cause of death.

In some countries biochemical tests for the detection of RNA tumour viruses are applied to new cell substrates. As the reliability of such tests becomes generally accepted consideration should be given to the inclusion of the tests in national requirements.

3.1.2.3 Cell cultures. Rabies virus used in the production of seed virus or vaccine shall be propagated in cell cultures approved by the national control authority.

Cell cultures shall be made from tissues derived from healthy animals.

\textsuperscript{2} This strain is available to laboratories, on request and with the approval of the national authorities, from the World Health Organization, Geneva, Switzerland.
All information on the source and method of preparation of the cell culture system used shall be available to the national control authority. Where applicable, details of any prophylactic and diagnostic measures to which the animals serving as a source of tissue may have been subjected and data showing freedom from infectious agents by the monitoring of antibody production shall be provided.

If chick embryo tissue is used for the propagation of rabies virus, the eggs used as a source of tissue shall be derived from flocks free from Salmonella infections, Mycobacterium avium, and other agents pathogenic for chickens.

If dog kidney tissue is used for the propagation of rabies virus, it shall be obtained either from dogs that are in overt good health and have been maintained in quarantine in vermin-proof quarters for a minimum of six weeks, having had no exposure to nonquarantined dogs or other animals throughout the quarantine period, or from dogs born in quarantine, provided they have been kept in the same type of quarantine continuously from birth.

In some countries the dogs are kept in quarantine for six months. Each dog should be examined periodically during the quarantine period and preferably shown to be free from antibodies to known canine viruses and, at the time of use, autopsied by a pathologist qualified in, or a physician or veterinarian having experience with, diseases of dogs, for signs of disease—e.g., tuberculosis, infectious canine hepatitis, canine distemper, rabies, leptospirosis, and other diseases of dogs. If any such signs or significant pathological lesions are observed, tissue from such animals should not be used.

If fetal bovine kidney tissue is used for the propagation of rabies virus it shall be obtained from the fetuses of cows that are in overt good health and have passed the pre- and postmortem examinations.

Kidney tissue should be obtained from fetuses of cows that have not been used previously for purposes involving infectious agents. If any signs of illness or significant pathological lesions are observed in pregnant cows the fetal tissue from such animals should not be used.

Special attention should be given to foot and mouth disease, brucellosis, Q fever, leptospirosis, bovine virus diarrhoea, infectious bovine rhinotracheitis, and tuberculosis, but in some areas other diseases of cattle may also have to be considered.

If hamster kidney tissue is used for the propagation of rabies virus, only hamsters of a strain approved by the national control authority shall be used as a source of tissue.
The animal stock should be free from infection with mycoplasma, *Mycobacterium tuberculosis*, lymphocytic choriomeningitis virus, reovirus, and cytomegalovirus, and from microorganisms pathogenic for hamsters. The parents of animals to be used as a source of tissue should be maintained in quarantine in vermin-proof quarters for a minimum of three months. Neither the parent hamsters nor their progeny should previously have been used for experimental purposes involving infectious agents.

Special attention should be given to RNA tumour viruses. Tests for absence of reverse transcriptase are recommended.

If *human diploid cells* are used for the propagation of rabies virus they shall be those approved by and registered with the national control authority. The cells shall have been characterized with respect to their genealogy, growth characteristics, viability during storage, and karyology, and they shall have been shown, by tests in animals and eggs, to be free from detectable adventitious agents (see Part C).

3.1.2.4 *Serum used in cell culture medium*. Serum used for the propagation of cells shall be tested to demonstrate freedom from bacteria, fungi, and mycoplasma, according to the requirements given in Part A, sections 5.2 and 5.3 of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (I, p. 49), as well as freedom from pathogens of the species of origin of the serum, by methods approved by the national control authority.

In some countries sera are examined for freedom from phage.

3.1.3 *Virus seed lot system*

The preparation of rabies vaccine shall be based on the use of a virus seed lot system. A working virus seed lot shall be not more than 5 passages removed from the master seed lot, which has been thoroughly characterized. Vaccines shall be made from a working seed lot without further intervening passage. Seed lots shall be maintained either in the dried or in the frozen form and be stored separately. If frozen, the seed shall be kept continuously at a temperature below −60°C.

Seed lots shall have been shown, to the satisfaction of the national control authority, to be capable of yielding vaccine that meets all these requirements.
3.1.4  Test on virus seed lots

Each seed lot shall be identified as rabies virus by methods approved by the national control authority.

3.1.4.1 Test on seed lots for vaccines produced in animal and embryonated duck eggs.

(1) Freedom from bacteria, fungi, and mycoplasmas
Each seed lot shall be tested for bacterial, mycotic, and mycoplasmal contamination by appropriate tests according to the requirements of Part A, section 5.2 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (I, p. 49).

(2) Virus content
A titration of virus content of each seed lot shall be made.

Such titrations may be done by the intracerebral inoculation of suitable dilutions in mice. The mice are observed for 14 days. The virus activity of the seed lot should be such that all mice are killed when so inoculated with 0.03-ml quantities of a dilution of not less than $10^{-4}$.

3.1.4.2 Test on virus seed lots for vaccines produced in cell cultures. In addition to the tests described in Part A, section 3.1.4.1, the master seed lot and each working seed lot used for the production of vaccine in both primary and diploid cell cultures shall be tested for Mycobacterium tuberculosis and mycoplasmas. Further, each seed lot shall be tested for adventitious agents in animals as described in this section and in cell cultures according to the tests specified in Part A, section 3.2.3. For these tests the virus shall be neutralized by a monospecific antirabies serum.

The individual tests on the seed virus should be so designed that they satisfy the requirements of the national control authority.

After neutralization of the rabies virus by hyperimmune serum the material shall be inoculated into animals and cell cultures as described below.

(1) Tests in suckling mice
A sample shall be tested for the presence of adventitious agents pathogenic to mice by intracerebral inoculation of 0.01 ml and intraperitoneal inoculation of at least 0.1 ml of the suspension into at least
10 mice less than 24 hours old originating from more than one litter. The mice shall be observed daily for at least 14 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be examined for evidence of viral infection; this shall be done macroscopically by direct observation and the tissues shall be examined microscopically and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least 5 additional suckling mice, which shall be observed daily for 14 days.

In some countries, in addition, a blind passage is made of a suspension of the pooled emulsified tissue (minus skin and viscera) of all mice surviving the original 14-day test.

The seed passes the test if at least 80% of the mice originally inoculated remain healthy and survive the observation period and if none of the mice shows evidence of infection with any adventitious agent attributable to the seed.

(2) Test in adult mice

A sample shall be tested for the presence of adventitious agents pathogenic to mice by intracerebral inoculation of 0.03 ml of the suspension, intraperitoneal inoculation of at least 0.25 ml and inoculation of 0.01 ml into the footpad in at least 20 adult mice, each weighing 15–20 g. The mice shall be observed for at least 4 weeks. All mice that die after the first 24 hours of the test or that show signs of illness shall be examined for evidence of viral infection; this shall be done macroscopically by direct observation as well as by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least 5 additional mice, which shall be observed for 3 weeks.

The seed passes the test if at least 80% of the inoculated animals remain healthy and survive the observation period and if none of the mice shows evidence of infection with any adventitious agent attributable to the seed.

(3) Test in guinea-pigs

A sample shall be tested for the presence of Mycobacterium tuberculosis or other adventitious agents by intraperitoneal inoculation of 5.0 ml into each of at least 5 guinea-pigs, each weighing 350–500 g. The animals shall be observed for at least 42 days. All guinea-pigs that die after the first 24 hours of the test or that show signs of illness shall be examined macroscopically and the tissues shall be examined both microscopically and culturally for evidence of infection with
Mycobacterium tuberculosis. Animals that survive the observation period shall also be examined by autopsy macroscopically for evidence of infection with Mycobacterium tuberculosis.

The seed passes the test if at least 80% of the inoculated guinea-pigs remain healthy and survive the observation period and if none of the animals shows evidence of infection with Mycobacterium tuberculosis or any other adventitious agent attributable to the seed.

The test may be made using the pellet from 100 ml of centrifuged bulk suspension resuspended in 30 ml of the supernatant. It is desirable to record the rectal temperature of the animals daily during the first 3 weeks. In some countries an in vivo test approved by the national control authority is used instead of the test in guinea-pigs.

(4) Tests in cell cultures

The neutralized seed virus shall be tested for freedom from extraneous viruses in cell cultures. The cell culture systems shall be: (i) that used for the growth of virus but not the same batch of cells as that used for the production of virus growth; and (ii) human cells. If the virus is grown in human diploid cells the cells used for testing shall not be from the same cell line.

In some countries the kidney cells of Cercopithecus or Erythrocebus monkeys are used.

Ten ml of the neutralized seed virus shall be tested in each cell system and incubated at 35-37°C for 14 days. At day 7 and day 14 a subculture shall be made of 10% of the pooled supernatant fluids and each of the subcultures incubated for 14 days.

The cells shall be observed microscopically for any cytopathic changes. At the end of the observation period, tests for haemadsorbing viruses shall be made (see Part A, section 3.2.3.1).

For the tests to be valid, at least 80% of the culture vessels shall be available and suitable for evaluation at the end of the observation period. For the seed virus to be satisfactory, no cytopathic changes shall occur and no extraneous agent shall be detected when the cells or fluids are tested according to the tests specified in Part A, section 3.2.3. Control cell culture shall be included in the tests.

3.2 Production of vaccine

Penicillin and streptomycin preparations shall not be used at any stage of manufacture of the vaccine unless the antibiotics are thor-
oughly washed from the cells. before these are seeded with virus, or eliminated from the virus suspension by a purification process.

3.2.1 Vaccines produced in neural tissue

The animals intended for production shall be kept in quarantine under veterinary supervision for at least two weeks prior to inoculation of the seed virus, except in the case of suckling animals, when this requirement shall apply to the mothers. Only animals free from any sign of disease shall be used. Seed virus shall be inoculated intracerebrally. Methods for inoculation and harvesting approved by the national control authority shall be used.

- While virus is always inoculated intracerebrally, the technique used varies with the species of animal. A satisfactory technique is one that consistently produces paralysis in the inoculated animals but does not introduce other infection.

- In order to obtain the maximum virus titre, neural tissues from inoculated animals, apart from suckling animals, should be harvested on an individual basis when the animal shows signs of advanced rabies.

- If suckling animals are used, the dose and date of inoculation should allow for harvesting of neural tissue before neuroallergenic activity becomes demonstrable. This can be done for the animal species and particular strain used for vaccine production by immunizing guinea-pigs with neural material suspended in complete Freund adjuvant. It is essential that positive and negative controls should be included in the test. On the basis of the results of the test, the period can be assessed during which acceptable material can be harvested. The time of harvest used by some production laboratories is 8 days for mice, 6 days for rabbits, and 7-11 days for rats.

Neural tissue shall not be taken from dead animals, whether death is due to rabies or to other causes.

All animals used in the production of vaccine should be examined by autopsy after the removal of neural tissue. If evidence of tuberculosis or any neural disease other than rabies is found, the nerve tissue from the animal should be discarded, or if nerve tissues have been pooled, the pool containing nerve tissue from such an animal should be discarded. If there is evidence of a communicable disease among the animals, the viral harvest from that group should be discarded.

When other than suckling animals are used, the tissue harvested from each animal shall be kept separate until completion of the steril-
ity test (Part A, section 3.3.1). When suckling animals are used, the harvest composed of a pool of tissue from a group of animals inoculated at the same time and harvested together shall similarly be kept separate until completion of the sterility test.

3.2.2 Vaccines produced in embryonated eggs

The eggs shall be derived from healthy flocks free from microorganisms known to be pathogenic for man and other pathogenic microorganisms and viral agents naturally occurring in ducks.

Such agents include *Salmonella pullorum*, *Mycobacterium* spp., mycoplasma, and avian leucosis viruses. If eggs are used from flocks that have not been shown to be free from avian leucosis viruses and mycoplasma, the method of inactivation used should have been shown, to the satisfaction of the national control authority, to be capable of killing these organisms.

After the eggs have been incubated for a suitable period they shall be inoculated with seed virus. After further incubation for a suitable period, the living embryos shall be harvested with aseptic precautions. Embryos inoculated at the same time and harvested together may be pooled and the viral harvest kept separate until completion of the sterility test (Part A, section 3.3.1).

3.2.3 Vaccines produced in cell cultures

At least 5% of the cell suspension (not less than 500 ml) at the concentration employed for seeding vaccine production cultures shall be used to prepare control cultures.

In some countries in which the technology of large-scale production has been developed, the national control authority should determine the size of the cell sample to be examined and the control methods to be applied.

The treatment of cells set aside as control material shall be similar to that of the production cell cultures, but they shall remain uninoculated as control cultures for the detection of extraneous viruses.

These control cell cultures shall be incubated under the same conditions as the inoculated cultures for at least two weeks or until the time of the last harvest of the production cultures—whichever is the later—and shall be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the
control cell cultures shall have had to be discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures shall be examined for degeneration caused by an infectious agent. If this examination, or any of the tests specified in this section, shows evidence of the presence in a control culture of any adventitious agent, the rabies virus grown in the corresponding inoculated cultures shall not be used for vaccine production.

3.2.3.1 Tests for haemadsorbing viruses. At the end of the observation period, cells comprising 25% of the control cells shall be tested for the presence of haemadsorbing viruses, using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage shall not have exceeded 7 days, and the temperature of storage shall have been in the range of 2–8°C.

This test is usually made using guinea-pig red cells. In some countries the national control authority requires that tests for haemadsorbing viruses should be made, in addition, using other types of red cells, including those from humans (blood group O), monkeys, and chickens (or other avian species).

All tests should be read after incubation for 30 min at 0–4°C and again after a further incubation for 30 min at 20–25°C. The test with monkey red cells should be read once more, after yet another incubation for 30 min at 34–37°C.

3.2.3.2 Tests for other extraneous agents. At the time of the last harvest of the production cultures, or 14 days after the day of inoculation of the production cultures with seed lot virus—whichver is the later—a sample of the pooled fluids shall be taken from each group of control cultures. Ten ml of each pool shall be tested in the same cells, but not the same batch of cells as that used for the production of virus growth, and additional 10-ml samples of each pool shall be tested in human cells and at least one other sensitive cell system. If the vaccine is produced in human diploid cells, the cells used for testing must not be from the same strain.

The inoculated cultures shall be incubated at a temperature of 35–37°C and shall be observed for a period of at least 14 days.

Some national control authorities require that at the end of this observation period a subculture shall be made in the same cell culture system and observed for at least 7 days. Furthermore, some national control authorities require that these cells should be tested for the presence of haemadsorbing viruses.
For the tests to be valid, at least 80% of the culture vessels shall be available and suitable for evaluation at the end of the respective test periods.

If any cytopathic changes occur in any of the cultures, the virus harvests produced from the batch of cells from which the control cells were taken shall be discarded.

3.2.3.3 Additional tests on control cells if avian embryo cells are used for production. A sample of the control fluid taken at the end of the observation period of the control cell cultures shall be tested for avian leucosis viruses and adenoviruses.

In some countries the complement fixing test (COFAL) is used for detecting avian leucosis viruses, and liver or kidney cell cultures of embryos are used for detecting adenoviruses.

Only those cells shown to be free from contamination may be used.

3.2.3.4 Additional tests on control cells if human diploid cells are used for production. Cells sufficient for chromosome monitoring (Part C, section 3.1.3) and for preparing control cultures (Part C, section 3.2) shall be taken from the pooled material removed from each culture vessel not earlier than two population doublings preceding the doubling level at which cells are to be inoculated with vaccine virus.

3.2.3.5 Additional tests on control cells if other cell cultures are used. When other cell cultures are used for the growth of rabies virus, additional tests for the detection of viruses specific for the host species shall be included. Such tests shall, to the satisfaction of the national control authority, be suitable for the purpose.

3.3 Control of bulk material

3.3.1 Sterility tests of the viral harvest

A sample removed from each viral harvest shall be tested for bacterial and fungal contamination by appropriate tests according to the requirements of Part A, section 5.2 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (I, p. 48). Any viral harvest in which contamination is detected shall be discarded.
3.3.2 Pooling of viral harvests

Only viral harvests satisfying the requirements for sterility given in Part A, section 3.3.1 of these requirements shall be pooled for bulk material.

In some countries the viral harvests are tested for the presence of extraneous viruses after neutralization of rabies virus (see Part A, section 3.1.4.2). The tests should be approved by the national control authority.

3.3.3 Homogenization of vaccine made in neural tissue

The apparatus used for homogenizing the brain tissue and embryos shall be of such a design as to prevent any escape of aerosols. The apparatus shall be housed in a safety cabinet.

The grinding and blending of tissues should be done at as low a temperature as possible to avoid destruction of virus.

Neural tissue vaccines should be prepared in such a way that a single dose for man in the immunization course is contained in not more than 2 ml of a 5% nerve tissue suspension or its equivalent—e.g., 1 ml of a 10% suspension.

In some countries it is required that a sample of the homogenized material should be titrated for living virus content.

3.3.4 Inactivation procedure

Methods and agents used for inactivation shall be approved by the national control authority. For vaccines made in neural tissue, inactivation shall be initiated immediately after homogenization. For vaccines made in cell cultures, inactivation shall be commenced immediately after clarification and filtration or purification (if a purification process is included). The method used shall be demonstrated to be consistently effective in the hands of the manufacturer. The inactivation process shall also have been shown, to the satisfaction of the national control authority, to be capable of inactivating mycoplasma, as demonstrated by in vitro tests. In the case of vaccine produced in embryonated eggs the method shall also be shown to inactivate avian leucosis viruses, as demonstrated by tests in tissue culture, or, in the case of vaccine produced in the brain of suckling animals, any adventitious agent that may be present, as demonstrated by tests using tissue culture or by animal inoculation. The inactivation of the virus must be completed within one half of the total inactivation time.
Various methods for inactivating rabies virus have been used with success. The concentration of the inactivating chemical, the temperature, and the length of time necessary for inactivation must be established for the particular type of vaccine being manufactured. A widely used agent is phenol, generally at a concentration ranging from 0.5% to 1% and at a temperature of 20–30°C for several days until complete inactivation has occurred, as demonstrated by the results of the test specified in Part A, section 3.3.6. Etherized vaccines are produced in some countries by combining the action of ether and phenol in the inactivation procedure. Beta-propiolactone (BPL) and tri-n-butyl phosphate have also been used. Satisfactory vaccines may be prepared by treating 10% neural tissue homogenates or virus suspensions from tissue culture at 20°C with a concentration of 1:3500 to 1:5000 BPL for 24 h or until complete inactivation has occurred, as demonstrated by the results of the test specified in Part A, section 3.3.6.

Ultraviolet light irradiation has also been used, but the equipment required and the procedures involved make it difficult to prepare vaccine in large volumes. The dosage range and time of application needed to accomplish complete inactivation of the virus without reducing antigenicity are critical, but when the radiation dose is regulated properly, highly antigenic vaccines may be prepared. The time required for inactivation is short compared with that needed when chemical methods are used, and hence the vaccine may be kept at a low temperature throughout; this also aids in conserving antigenicity.

Vaccines may be freeze-dried. For the best results the lapse of time from inactivation to initiation of the freeze-drying cycle should be kept to a minimum.

### 3.3.5 Concentration and purification

If concentration and purification of the virus harvest are carried out, the methods used shall be approved by the national control authority. The manufacturer shall show, to the satisfaction of the national control authority, that concentration has been effected.

The potency test is a useful measure of the degree of concentration achieved.

### 3.3.6 Test for effective inactivation

Each bulk material shall be tested in mice for inactivation of virus prior to the addition of preservatives and other substances. The tests shall be approved by the national control authority.
The test should be performed with undiluted bulk material injected intracerebrally into at least 20 mice, each weighing between 15 and 20 g. In some countries tests are done also in rabbits or guinea-pigs.

In some cases the concentration of inactivating agent or tissue in undiluted bulk material may be toxic to the test animals. In this case the test should be performed on final bulk material, which may be diluted, if necessary, but the dilution should be not more than 1:2.

At least 20 mice and 3 rabbits should be used. If the virus was propagated in an animal other than the rabbit, consideration should be given to using the production species rather than the rabbit.

For vaccine produced in cell cultures the rabies virus amplification test shall be used in the cell culture of origin to test for the presence of live virus. At least 25 ml of virus pool corresponding to 25 human doses shall be tested in this way (8) or by another method of similar sensitivity.

The bulk material passes the test if the product has been shown, to the satisfaction of the national control authority, to be free from residual live virus.

In some countries the cell cultures are subcultured once or twice in order to increase the sensitivity of this test.

3.4 Preparation and control of final bulk

3.4.1 Preservatives and other substances added

In preparing the final bulk, only the preservatives or other substances 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 amounts used.

If phenol has been used for inactivation, its concentration in the final bulk shall be such that it will not exceed 0.25% in the final product.

If beta-propiolactone has been used for inactivation, the procedure shall be such that there is no detectable amount of the chemical in the final bulk. The test method used shall be approved by the national control authority.

No antibiotics shall be added to rabies vaccine for human use.
3.4.2 Potency test on the final bulk

The manufacturers may wish to carry out a potency test on the final bulk. If this test is done, however, it will not eliminate the need for the test for potency that must be performed on the final product.

For vaccines made in cell cultures a suitable test to be applied to the bulk is the antibody binding test (9).

3.4.3 Sterility tests

Each final bulk shall be tested for sterility according to the requirements given in Part A, section 5.2 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (I, p. 48).

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) (7, p. 16) shall apply, with the addition of the following directives:

Containers of dried vaccine shall be hermetically sealed under vacuum or after filling with pure, dry, oxygen-free nitrogen or any other gas not deleterious to the vaccine. All containers shall be tested for leaks and all defective containers shall be discarded.

Generally only single-dose containers are used.

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 an appropriate method.

The test for potency, as described in Part A, section 5.4, may serve as an identity test.

5.2 Sterility tests

Each filling lot shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5.2 of the
revised Requirements for Biological Substances (General Requirements for the Sterility of Biological Substances) (I, p. 48).

5.3 Innocuity tests

Each filling lot shall be tested for abnormal toxicity by appropriate tests in mice and guinea-pigs, using parenteral injections. The test procedures shall be those approved by the national control authority.

5.4 Potency test of vaccine in final containers

A test for potency shall be made on each filling lot. Before the test is made, dried vaccine shall be reconstituted to the form in which it is to be used in man.

The test shall be one in which mice are immunized and subsequently challenged with rabies virus and shall be made in parallel with a reference vaccine. The challenge strain\(^\text{3}\) and reference vaccine, as well as the test procedure used, shall be those approved by the national control authority (see Part B, section 1).

Reproducibility of the test depends in part on the strain of rabies virus used for challenge and its maintenance in a large homogeneous working pool kept below \(-60^\circ\text{C}\). The strain of mice may also affect reproducibility.

When the NIH test is used, the potency relative to a reference preparation is determined (10).

A reference preparation with an activity calibrated in international units by comparison with the third International Reference Preparation of Rabies Vaccine is included in each test. The potency of the vaccine in international units is then determined by comparing its activity with that of the reference preparation.

For vaccines given in a six-dose schedule the potency should be at least 2.5 IU per single human dose calculated as the geometric mean of potency values found in two or more tests. The confidence limits of the resulting value should be within a certain interval, to be decided by the national control authority.

When the Habel test (1I) is used for vaccines made in brains or avian embryos, inclusion of the reference vaccine (see Part A, section 1:2) would enable the sensitivity of the test system to be monitored in the testing of successive batches.\(^\text{4}\)

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\(^3\) A suitable challenge strain, CVS, is available to laboratories, on request and with the approval of the national authorities, from the World Health Organization, Geneva, Switzerland.

\(^4\) The reconstituted International Reference Preparation of Rabies Vaccine, when injected into mice in volumes of 0.25 ml of a strength corresponding to a 0.5% suspension, has been shown to protect against more than 10,000 LD\(_{50}\) of CVS rabies virus under the conditions of the test.
5.5 Stability test for freeze-dried vaccine

The method of production of vaccine shall be such that stable vaccine is produced as shown by an accelerated degradation test. The test used shall be approved by the national control authority.

In some countries stability is ascertained by testing samples throughout the shelf-life of the vaccine.

The test for potency (see Part A, section 5.4), made after the storage of samples for 4 weeks at 37°C, is suitable. In order to pass the test the lot should retain minimum potency, as defined in Part A, section 5.4.

In some countries each lot of vaccine must be subjected to the stability test, whereas in others the test is required only for the initial licensing lots to show consistency of production.

5.6 Residual moisture tests on freeze-dried vaccine

In the case of dried vaccine it is advisable to test for residual moisture, as a guide to the maximum content allowable for the stability of the product.

With some vaccines it is possible to dry the product to less than 1% residual moisture without impairing its stability and potency. However, depending on the type of stabilizer present, higher values may be accepted by the national control authority.

5.7 Inspection of final containers

Each container in each filling lot shall be inspected, and those showing abnormalities shall be discarded.

5.8 Test for pyrogenicity

In some countries vaccines prepared in cell cultures are tested in rabbits for the presence of pyrogens. The limits of the test should be determined by the national control authority.

The Limulus amoebocyte lysate test can also be used.

5.9 Measurement of serum concentration

As all vaccines made in tissue culture are concentrated and, depending on the method of concentration, the small quantities of serum present in the tissue culture fluids may also be concentrated, some countries require the concentration of serum in the final vaccine to be measured. The national control authority should determine the maximum permissible limit.
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) (7, p. 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) (7, p. 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) (7, p. 18) shall apply, with the addition of the following directive:

The leaflet accompanying the package shall include the following information:
- the tissue and animal species in which the vaccine was prepared;
- the method used for inactivating the virus; and
- if the vaccine is in the dried form, a statement that, after its reconstitution, it shall be used as soon as possible or stored at $5 \pm 3^\circ C$ and discarded at the end of the day.

9. DISTRIBUTION AND SHIPPING

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) (7, p. 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) (7, p. 19) shall apply.

10.1 Storage conditions

Rabies vaccine (for human use) shall be stored at a temperature of 5 ± 3 °C.

10.2 Expiry date

The expiry date shall be based on data submitted by the manufacturer and shall be determined by the national control authority.

It has been usual to allow an expiry date of 6–12 months for the liquid vaccine and 18–24 months for the dried vaccine, the period concerned starting from the last satisfactory potency assay.

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) (7, p. 19) shall apply.

The national control authority shall give directions to manufacturers concerning the strain of rabies virus\textsuperscript{5} to use for the production of vaccine.

The national control authority shall provide or approve the strain for challenge\textsuperscript{4} and the reference vaccine for use in the potency test (Part A, section 5.4).

2. RELEASE AND CERTIFICATION

A vaccine lot shall be released only if it fulfills Part A of these requirements.

\textsuperscript{5} The Pasteur strain for vaccine production and the CVS virus are available to laboratories, on request and with the approval of the national authorities, from the World Health Organization, Geneva, Switzerland.
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 whether or not the lot of vaccine in question meets all national requirements as well as Part A of the present requirements. The certificate shall further 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 rabies vaccine (for human use) between countries.

PART C.
REQUIREMENTS FOR HUMAN DIPLOID CELLS USED FOR VIRUS VACCINE PRODUCTION

The following requirements are applicable to the cell substrate for virus vaccine production where production is based on a cell seed system. The tests so far have been formulated for human diploid cells but any cell bank and cell seed system shall comply with similar requirements as appropriate.

1. DEFINITIONS

1.1 Terminology

Cell seed: a quantity of cells derived from a single human tissue stored frozen at \(-70^\circ\text{C}\) or below in aliquots, one of which would be used for the production of a manufacturer's working cell bank.

Manufacturer's working cell bank (MWCB): a quantity of cells derived from a single ampoule of the cell seed and of uniform composition stored frozen at \(-70^\circ\text{C}\) or below in aliquots, one of which would be used for the production of each single harvest.

In normal practice such a seed culture (or ampoule) is issued to manufacturers at or near the eighth population doubling level (PDL). This is expanded by serial subculture up to a PDL selected by the manufacturer at which point the cells are combined into one or more pools and preserved cryogenically to form the
MWCB. One or more of such ampoules from a pool would be used for the production of a single harvest.

Production cell culture: a collection of cell cultures at the population doubling used for virus growth that have been derived from a single ampoule of the MWCB.

2. GENERAL MANUFACTURING REQUIREMENTS

The general requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (7, p. 11) shall apply, with the addition of the following directive:

No cell cultures other than those approved by the national control authority for the production of appropriate vaccine shall be introduced or handled in the production area.

3. PRODUCTION CONTROL

3.1 Cell seed

The utilization of human diploid cell cultures for vaccine manufacture shall be based on the cell seed system. Early population doubling of diploid cell cultures shall be subcultured to a population doubling which is convenient for the preparation of a cell seed.

The cell seed used for the production of virus vaccine shall be that approved by and registered with the national control authority. The accepted cell strain from which the cell seed has been derived shall have been characterized with respect to genealogy, growth characteristics, genetic markers (HLA), virus susceptibility, storage conditions, and karyology, and it shall have been shown, by tests in animals, eggs, and cell culture, to be free from detectable adventitious agents.

These data shall be made available to the national control authority. Each manufacturer shall show, to the satisfaction of the national control authority, that the cell substrate propagated from the accepted cell strain and laid down as a working cell bank conforms with the tests outlined in this section for freedom from extraneous agents, by tests in animals for lack of tumorigenicity, for normal karyology throughout approximately the first two-thirds of its normal life-span, and for identity.
3.1.1 Tests in animals and eggs for extraneous agents.

The tests in animals for extraneous agents shall include the inoculation of each of the following groups of animals with cells by the intramuscular route, using at least $10^7$ viable cells divided equally between the animals in each group:
- 2 litters of suckling mice, comprising at least 10 animals, less than 24 hours old;
- 10 adult mice;
- 5 guinea-pigs; and
- 5 rabbits.

At least $10^6$ viable cells shall be injected also into the allantoic cavity of each of 10 embryonated chicken eggs 9–11 days old.

The animals shall be observed for at least four weeks and the embryonated chicken eggs shall be examined after not less than three days. Any animals that are sick or show any abnormality shall be investigated to establish the cause of illness. The allantoic fluids shall be tested with guinea-pig and chick or other avian red cells for the presence of haemagglutinins.

The cells are suitable for vaccine production if at least 80% of the animals or eggs inoculated with the cells remain healthy and survive the observation period, and none of the animals or eggs show evidence of the presence in the cell cultures of any extraneous agent.

3.1.2 Tumorigenicity

The cells at the production level shall also be shown to be free from potential tumorigenicity by appropriate tests in animals approved by the national control authority.

Suitable tests using immunosuppressed animals may be made as follows. Approximately $10^6$ viable cells obtained from cultures at the same passage level as those used for vaccine production are injected into: (a) newborn mice or hamsters treated with antilymphocyte serum; or (b) athymic mice (nu/nu genotype); or (c) thymectomized mice irradiated and bone marrow reconstituted (T-B+). Some of the same group of animals should be inoculated with a similar dose of HeLa or KB cells, and it should be shown that tumour formation is caused by the inoculation of the neoplastic tissue, thus demonstrating the ability of the strain.
of animals to give rise to tumours. The animals should be observed for not less than three weeks. Any other test using animals treated with immunosuppressive agents and with equal sensitivity to neoplastic cells may be used.

Only those cell seeds shown not to be tumorigenic shall be used.

3.1.3 Chromosomal characterization and monitoring

3.1.3.1 Chromosomal characterization. At least four samples shall be examined, as described in Part C, section 3.1.3.2, at approximately equal intervals over the life-span of the cell line during serial cultivation. Each sample shall consist of 1000 metaphase cells.

It is also recommended that photographic reconstruction should be employed in the preparation of chromosome-banded karyotypes of 50 metaphase cells per 1000-cell sample using either G-banding or Q-banding techniques. This constitutes 5% of the sample and no specific limits for acceptability are yet recommended. The incidence of karyotypic abnormalities (pseudodiploidy, inversions, translocations, etc.) that are detectable with the greater resolution provided by banding should be evaluated when a larger data base than is at present available has been accumulated.

3.1.3.2 Chromosomal monitoring—preparation and testing. For the determination of the general character of each pool in the MWC8B, a minimum of 500 cells in metaphase shall be examined at the production level or at any passage thereafter for frequency of polyploidy and for exact counts of chromosomes, frequency of breaks, structural abnormalities, and other abnormalities such as despiralization or marked attenuations of the primary or secondary constrictions.

For vaccine production, examination of the cells is usually made between the twenty-seventh and thirty-third population doubling. The national control authority should determine the level of cell population doubling allowable.

For cells examined in metaphase the upper limits* of acceptability (upper fiducial limits at 95% (Poisson)) for abnormalities for a 1000- and a 500-cell sample are as follows:

*These upper limits are based on extensive experience with the examination of WI-38 and MRC5 cells reported to and examined by the Ad Hoc Committee on Karyological Controls of Human Cell Substrates, which met in 1978 at Lake Placid, NY, USA. These values will not necessarily be applicable if another human cell strain is used.

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<table>
<thead>
<tr>
<th>Abnormality</th>
<th>1000 cells</th>
<th>500 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatid and chromosome breaks</td>
<td>47/1000</td>
<td>26/500</td>
</tr>
<tr>
<td>Structural abnormalities</td>
<td>17/1000</td>
<td>10/500</td>
</tr>
<tr>
<td>Hyperploidy</td>
<td>8/1000</td>
<td>5/500</td>
</tr>
<tr>
<td>Hypoploidy</td>
<td>180/1000</td>
<td>90/500</td>
</tr>
<tr>
<td>Polyploidy</td>
<td>30/1000</td>
<td>17/500</td>
</tr>
</tbody>
</table>

All cells showing abnormalities shall be subjected to detailed examination, and records shall be maintained of the detailed criteria applied to particular abnormalities evaluated in the karyotype analysis.

Stained slide preparations of the chromosomal monitoring of the working cell bank pool, or photographs of these, shall be maintained permanently as part of the record of the batch of vaccine and for monitoring successive batches made from that cell pool.

It is desirable that a portion of the sample of pooled cell substrate removed from the culture vessels should be stored frozen so as to retain viability. This would be available for future reference for karyology or for any other purpose relating to the batch of vaccine.

Only those cell pools of the MWCB that have normal karyology shall be used for vaccine production.

3.1.4 Identity test of the cells

In some countries tests for characterizing HLA surface antigens are carried out in addition to chromosome monitoring.

3.2 Production of cell culture

At least 5% of the cell suspension (not less than 500 ml) of the concentration employed for seeding the vaccine production cultures shall be used to prepare control cultures.

In some countries in which the technology of large-scale production has been developed, the national control authority should determine the size of the sample of cells to be examined.

The treatment of cells set aside as control material shall be similar to that of the production cell cultures, but they shall remain un inoculated as control cultures for the detection of extraneous viruses.

These control cell cultures shall be incubated under the same conditions as the inoculated cultures for at least two weeks or until the time of the last harvest of the production cultures—whichever is the
later—and shall be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures shall have had to be discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures shall be examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section shows evidence of the presence in a control culture of any adventitious agent, the virus grown in the corresponding inoculated cultures shall not be used for vaccine production.

3.2.1 Test for haemadsorbing viruses

At the end of the observation period, cells comprising 25% of the control cells shall be tested for the presence of haemadsorbing viruses, using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage shall not have exceeded 7 days, and the temperature of storage shall have been in the range of 2–8 °C.

In some countries the national control authority requires that tests for haemadsorbing viruses should be made in addition, using other types of red cells, including those from humans (blood group IV, O), monkeys, and chickens (or other avian species). The cultures should be examined at 3–5 days and again at 12 days. All tests should be read after incubation for 30 min at 0–4 °C and again after a further incubation for 30 min at 20–25 °C. The test with monkey red cells should be read once more, after yet another incubation for 30 min at 34–37 °C.

3.2.2 Tests for other extraneous agents

Fourteen days after the day of initiating the control cells, or at the time of the last harvest from the production cultures—whichever is the later—a sample of the pooled fluids shall be taken at each period of collection from each group of control cultures. Ten ml of each pool shall be tested in the same cells, but not the same batch of cells as that used for the production of virus growth, and additional 10-ml samples of each pool shall be tested in human cells and at least one other sensitive cell system.

The inoculated cultures shall be incubated at a temperature of 35–37 °C and shall be observed for a period of at least 14 days.

Some national control authorities require that at the end of this observation period a subculture should be made in the same
cell culture system and observed for at least 7 days. Furthermore, some national control authorities require that these cells should be tested for the presence of haemadsorbing viruses.

For the tests to be valid, at least 80\% of the culture vessels shall be available and suitable for evaluation at the end of the respective test periods.

If any cytopathic changes occur in any of the cultures the virus harvests produced from the batch of cells from which the control cells were taken shall be discarded.

3.2.3 Identity test

At the production level the cells shall be identified as human by tests approved by the national control authority.

Suitable tests are isozymes, HLA, or other immunological tests or karyotype of at least one metaphase spread of chromosomes.

AUTHORS

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ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice and for supplying additional data relevant to these Requirements:
REFERENCES


Appendix

SUMMARY PROTOCOL FOR RABIES VACCINE (HUMAN) PRODUCTION AND TESTING

Identification of Final Lot

Name and address of manufacturer

Lot number of vaccine

Date of manufacture of final lot

Expiry date

Type of vaccine
(animal brain/eggs/cell culture)

Vaccine Virus Strain

Name and short description of history, origin, process of attenuation and adaptation

89
Date of preparation of master seed virus
Number of passages between isolation and master seed
Date of preparation of working seed
Number of passages between master and working seed

Virus Production Substrate

(Complete only the relevant part—i.e., A, B, C, D or E.)

A. Animal neural tissue (brains)

Animal species
Adult/weanling/suckling
Number of animals used
Age of animals
Quarantine period
Period between inoculation and harvest
Result of autopsy
Result of sterility test on harvested tissue (to be indicated by the terms "pass" or "fail")

B. Embryonated eggs

Animal species
Origin of eggs
Incubation period
Period between inoculation and harvesting
Number of eggs harvested
Result of sterility test
Other tests

C. Cell cultures/avian embryo

Amount of cell suspension used in vaccine production

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<table>
<thead>
<tr>
<th>Section</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of cell suspension used to prepare control cultures (ml)</td>
<td></td>
</tr>
<tr>
<td>Results of control tests</td>
<td></td>
</tr>
<tr>
<td>Test for haemadsorbing viruses</td>
<td></td>
</tr>
<tr>
<td>Tests for extraneous agents</td>
<td></td>
</tr>
<tr>
<td>Test for avian leukosis viruses</td>
<td></td>
</tr>
<tr>
<td>Test for adenoviruses</td>
<td></td>
</tr>
<tr>
<td>Other tests (sterility, mycoplasma, etc.)</td>
<td></td>
</tr>
<tr>
<td><strong>D. Cell cultures/human diploid or continuous</strong></td>
<td></td>
</tr>
<tr>
<td>Cells used for production</td>
<td></td>
</tr>
<tr>
<td>Authority by which cell seed was approved</td>
<td></td>
</tr>
<tr>
<td>Amount of cell suspension used in vaccine production</td>
<td></td>
</tr>
<tr>
<td>Amount of cell suspension used to prepare control cultures (ml)</td>
<td></td>
</tr>
<tr>
<td>Results of control tests</td>
<td></td>
</tr>
<tr>
<td>Test for haemadsorbing viruses</td>
<td></td>
</tr>
<tr>
<td>Tests for extraneous agents</td>
<td></td>
</tr>
<tr>
<td>Identity test of cells</td>
<td></td>
</tr>
<tr>
<td>Results of chromosome monitoring of cell seed at production level</td>
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</tr>
<tr>
<td>Other tests (sterility, mycoplasma, etc.)</td>
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</tr>
<tr>
<td><strong>E. Cell cultures/other cells</strong></td>
<td></td>
</tr>
<tr>
<td>Type of cell culture (including host species)</td>
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</tr>
<tr>
<td>Amount of cell suspension used</td>
<td></td>
</tr>
<tr>
<td>Amount of control suspension investigated (ml)</td>
<td></td>
</tr>
<tr>
<td>Results of control</td>
<td></td>
</tr>
<tr>
<td>Test for haemadsorbing viruses</td>
<td></td>
</tr>
<tr>
<td>Test for extraneous agents</td>
<td></td>
</tr>
<tr>
<td>Test for viruses specific for the host species</td>
<td></td>
</tr>
<tr>
<td>Other tests (sterility, mycoplasma, etc.)</td>
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</table>
### Virus Cultures

<table>
<thead>
<tr>
<th>Item</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number(s) of culture(s)</td>
<td></td>
</tr>
<tr>
<td>Date of inoculation of virus</td>
<td></td>
</tr>
<tr>
<td>Date of viral harvest</td>
<td></td>
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### Bulk Material

<table>
<thead>
<tr>
<th>Item</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbers of viral harvests included</td>
<td></td>
</tr>
<tr>
<td>Date of pooling</td>
<td></td>
</tr>
</tbody>
</table>

**Sterility test**

- Have all the harvests included been tested for sterility?  
- Results of these tests

### Control of inactivation

<table>
<thead>
<tr>
<th>Item</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nature of concentration and/or purification (if applied)</td>
<td></td>
</tr>
<tr>
<td>Method of inactivation</td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
</tr>
<tr>
<td>Test for inactivation—volume of material injected (including concentration)</td>
<td></td>
</tr>
<tr>
<td>Number of mice injected</td>
<td></td>
</tr>
<tr>
<td>Weight of mice</td>
<td></td>
</tr>
<tr>
<td>Duration of observation</td>
<td></td>
</tr>
<tr>
<td>Other animals (if used)</td>
<td></td>
</tr>
<tr>
<td>Result of tests</td>
<td></td>
</tr>
<tr>
<td>Result of virus amplification test (for cell vaccines)</td>
<td></td>
</tr>
<tr>
<td>Amount of vaccine tested (ml)</td>
<td></td>
</tr>
</tbody>
</table>

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Final Bulk

Preservatives, etc.
Concentration of phenol (if used)
Other preservatives (type and concentration)

Sterility tests
Date of test and result

Other tests (chemical, biochemical)
Type of test
Result

Test on Final Lot

1. Identity test
   Method used
   Result

2. Sterility tests
   Number of containers examined
   Method of test
   Date at start of test
   Date at end of test
   Result

3. Innocuity tests
   Mice
   Guinea-pigs
   Number of animals
   Route of injection
   Volume of injection
   Date of injection
   Date of end of test
   Result
4. **Chemical and biochemical tests**

<table>
<thead>
<tr>
<th>Type of test</th>
<th>Result</th>
</tr>
</thead>
</table>

5. **Potency test**

<table>
<thead>
<tr>
<th>Type of test</th>
<th>Date of test</th>
</tr>
</thead>
</table>

**Immunization of mice**

<table>
<thead>
<tr>
<th>Date of start of test</th>
<th>Reference vaccine (potency)</th>
<th>Challenge strain</th>
<th>Date of challenge</th>
<th>ED_{50} test vaccine</th>
<th>ED_{50} reference vaccine</th>
<th>Calculated IU/single human dose</th>
<th>Confidence limits</th>
<th>Results of other potency tests</th>
</tr>
</thead>
</table>

6. **Stability test for freeze-dried vaccine**

<table>
<thead>
<tr>
<th>Duration and temperature of incubation</th>
<th>Result</th>
</tr>
</thead>
</table>

7. **Residual moisture test for freeze-dried vaccine**

<table>
<thead>
<tr>
<th>Method used</th>
<th>Result</th>
</tr>
</thead>
</table>

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 (and, if HDC were used, Part C) of the WHO Requirements for Rabies Vaccine for Human Use.

Date

_____________________________

Signature

_____________________________

Name typed

_____________________________

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

The following International Requirements for Rabies Vaccine for Veterinary Use have been fitted into the framework adopted in the Requirements for Biological Substances Nos. 1–28, already published by WHO (I, p. 142), and, in drafting them, account has been taken of the opinions of consultants, and the regulations and requirements for the manufacture and control of rabies vaccine that have been
formulated in a number of countries, as well as information from both published and unpublished reports. In addition, comments have been received from a number of experts (see Acknowledgements, page 120).

GENERAL CONSIDERATIONS

Although the spread of rabies is associated with a variety of animal species, vaccination can prevent the disease in domestic animals, thus effectively eliminating these species as sources of human exposure. Nevertheless, the vast majority of cases of human rabies which occur, especially in the developing countries, are contracted through exposure to rabid dogs. In some countries cats are also important transmitters of rabies to man. Control of canine and feline rabies through effective vaccination thus appears to be potentially the most promising single practicable measure that can be taken to decrease the number of human cases worldwide. Infected cattle may also present a considerable danger to man, as the symptoms are less clear and may lead to confusion with other diseases.

Vaccines used in animals include a wide variety of types. Live attenuated vaccines are produced in cell cultures and in embryonated eggs. Inactivated vaccines are produced in cell cultures and in neural tissues of suckling or adult animals. Rabies vaccine for veterinary use may be prepared also in combination with other viral or bacterial vaccines.

A number of different manufacturing and testing procedures are in use in various countries. The procedures differ according to the type of vaccine—whether live or inactivated—the strain of virus, the species of animal or type of cell culture used for propagation of the virus, the method of inactivation, the preservatives and/or adjuvants added, the methods for testing potency, and the form in which the vaccine is used.

Because some inactivation procedures will destroy many contaminating viruses and mycoplasmas, a reduced range of tests may be permitted for inactivated vaccines in some countries. Similarly, in some countries, it is considered that the risks of pathogenic contaminants are reduced in vaccines which are produced in embryonated eggs or avian cells and which are to be used in mammals.

The passage histories of the different strains of virus being used for production are not all well documented. Such a strain should be one
known to produce vaccine that is antigenically active against classical rabies virus strains (i.e., those belonging to serotype 1 of the rabies subgroup of rhabdoviruses). While many strains have been derived from the original Pasteur fixed strain, others used for production have been isolated more recently from man or animals. Strains used for production of vaccine should be limited to what are termed “fixed” strains of virus. It is desirable that studies should be made in order to define qualitatively and quantitatively the properties of such a “fixed” strain. This is one that has a short, stable, and reproducible incubation period when injected intracerebrally into suitable animals. However, it can be demonstrated that strains in use at the present time differ considerably in their ability to produce rabies in experimental animals when inoculated by a route other than the intracerebral.

Strains used in the production of live attenuated vaccine must also show no virulence for the species in which they are used and should be apathogenic for man. (Not all strains fulfil this requirement under extreme conditions. Exposure to abnormally high concentrations may create problems, and the manufacturer should indicate whether post-exposure treatment is necessary.) It is also desirable that, particularly for live vaccines, studies should be made to establish appropriate genetic markers to characterize the virus used for production.

The use of the seed lot system has been specified, and since the International Standard for Anti-Rabies Serum is not made widely available it is recommended that a monospecific anti-rabies serum should be produced and made available by national control authorities as an aid to establishing the identity and purity of the seed virus.

In contrast to rabies vaccines for human use, animal vaccines are normally used only before exposure. It follows, then, that the duration of immunity is at least as important a consideration as the onset of immunity. Immunity should persist in all species for one full year after vaccination. In dogs, for example, a three-year duration of immunity is attainable and desirable. High egg passage (HEP) vaccines, commonly used in cats, do not always give sufficient immunity for a full year; such vaccines should not be used, or a guarantee for one year should not be given. Except where high titres of neutralizing antibodies are demonstrated, the efficacy of animal rabies vaccine must be proved by experimental challenge with street virus.

Neural tissue rabies vaccines have been in worldwide use for generations, and experience has indicated that they are effective. It is generally accepted that there is some risk of central nervous system involvement after administration of the vaccine prepared in the brains.
of adult animals. Such adverse reactions are essentially eliminated when the virus is grown in the brains of suckling animals in which myelin has not yet developed.

Care must be taken to ensure that live attenuated vaccines are used only in those species in which they have been thoroughly tested and proved safe. Some strains used in the production of live vaccines will elicit clinical signs of rabies in a relatively high percentage of animals of certain species. For example, low egg passage (LEP) Flury strain will induce rabies in cats, cattle, and in a variety of other less commonly vaccinated species. In very sensitive species (cats, cattle) inactivated vaccines are preferable. HEP Flury strain was found to be pathogenic for mustelids and the Street Alabama Dufferin (SAD) strain for a variety of other wild animals, especially rodents.

The development of new animal rabies vaccines is hindered by the difficulty and high cost of evaluating their efficacy and safety. To prove efficacy, test animals must be maintained for a minimum of one full year—and in some cases three years—between vaccination and challenge of immunity. This requirement, which, together with the extensive controlled studies and field trials, is necessary to establish in each species the safety of any new strain used in live vaccines, raises the development costs of rabies vaccines to exceptionally high levels.

The potency testing of animal rabies vaccine is of considerable concern. The potency test must be capable of discriminating between vaccines of different activity. Preferably, a single test should be applicable to all types of inactivated vaccine. The rabies virus strain used for challenge should be one of reproducible virulence for the test animal when given intracerebrally. In addition, the test should be reproducible and economical. A common reference preparation of vaccine is of importance in evaluating test results. Ideally, such a reference preparation should be protective in animals. Classically, for neural vaccines, potency has been based on the wet weight (mg) of neural tissue required for the protection of 50% of the test animals. However, in the case of purified vaccines that contain reduced amounts of host tissue, as well as in that of cell culture vaccines, the potency should be expressed on the basis of the dilution of vaccine (injected in a defined volume) protecting 50% of the test animals rather than on the basis of the tissue content. By this procedure a single reference vaccine may be used for routine testing of potency of all types of inactivated rabies vaccine. This has the additional advantage of providing a common basis for comparing the potency of the classical neural tissue vaccines, with which there have been many years of experience,
and the potency of the newer types of vaccine. The potency required to protect animals must be proved for each species for which the vaccine is recommended.

It is important also to consider the inclusion of an antibody response test in animals, since this can be used to discriminate between vaccines of different potencies.

Potency testing of individual serial lots of live vaccine may be limited to the determination of the quantity of viable virus in each dose, when this quantity has been shown to be effective in the host animal.

Tests for factors in the vaccine that may induce allergic encephalomyelitis have not been included in these requirements because no reliable techniques have been described. The degree of reproducibility of existing procedures has not been evaluated, and there is evidence that considerable variation in results can be expected. Existing tests, however, can be used to assess the period during which the factor causing allergic encephalomyelitis develops in the brains of young animals. Studies should continue to be encouraged for improving such tests as long as neural tissue vaccines are used.

The use of healthy animals has been specified in these requirements. National control authorities should pay attention to the problems of ensuring that the animals used are free from infectious agents that might contaminate rabies vaccine.

National authorities should also discourage the use of the brains of adult animals for the production of vaccine. A determined effort should be made to avoid this practice, either by the use of immature animals, such as suckling mice, or by the use of cell cultures.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements, so that, if an animal health administration so desires, these parts as they appear may be included in 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 rabies vaccine, it is recommended that a clause should be included permitting modifications of manufacturing requirements on the condition that it can 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 and/or used.

PART A.
MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be “Vaccinum rabiei vivum (ad usum veterinarium)” or “Vaccinum rabiei inactivatum (ad usum veterinarium)”. The proper names shall be the equivalent of the international names in the language of the country of origin.

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

1.2 Descriptive definition

Vaccinum rabiei vivum (ad usum veterinarium) is a freeze-dried preparation of rabies “fixed” virus grown in embryonated eggs or in cell cultures.

Vaccinum rabiei inactivatum (ad usum veterinarium) is a fluid or freeze-dried preparation of rabies “fixed” virus grown in the neural tissue of rabbits, sheep, goats, mice, rats or other animals, or in cell cultures, inactivated by a suitable method. Some inactivated vaccines may contain an adjuvant.

The preparations shall satisfy all the requirements formulated below.

1.3 International Reference Preparation and International Standard

The International Reference Preparation of Rabies Vaccine, established in 1978 (2, p. 15), is stored and distributed in ampoules containing freeze-dried rabies vaccine, prepared in human diploid cells and inactivated with beta-propiolactone. There is an activity of 10 IU per ampoule. This reference preparation is intended for the calibration of national reference preparations for use in tests of potency of inactivated rabies vaccines (see Part B, section 1). After reconstitution the International Refer-
ence Preparation may be stored for subsequent animal immuniza-
tions provided that the storage temperature is below -60 °C and
that the period of storage is not longer than one month.

The International Standard for Anti-Rabies Serum established
in 1955 (3. p. 11), is stored and distributed in ampoules con-
taining 86.6 IU. This standard is intended for use in the laboratory
assay of potency of antirabies immunoglobulin preparations used
in man. It can also be used for the assay of rabies antibodies in
man and animals.

The reference preparation and the standard 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.

1.4 Terminology

*Master seed lot:* a quantity of virus that has been processed together
and has a uniform composition. It is used for vaccine preparation or
for the preparation of further working seed lots.

*Working seed lot:* a working seed lot which is prepared from the
master seed lot and which shall be not more than 10 passages removed
from it.

*Cell seed:* a quantity of cells derived from a single human or ani-
mal tissue and of uniform composition, stored frozen at -70 °C or
below in aliquots, one of which would be used for the production of a
single harvest.

*Single viral harvest:* virus harvested from a single animal or from a
group of suckling animals or a group of embryonated eggs or cell
cultures inoculated at the same time and harvested together. The
virus in the harvest is without intervening passage from the working
seed lot.

*Bulk material:* a pool of single viral harvests before preparation of
the final bulk. It may be prepared from one or a number of viral
harvests and may yield one or more final bulks.

*Final bulk:* the finished biological preparation present in the con-
tainer from which the final containers are filled. It may be prepared
from one clarified bulk suspension or from a blend of clarified bulk
suspensions.

*Filling lot (final lot):* a collection of sealed final containers, dis-
persed from the same final bulk, that are homogeneous with respect
to the risk of contamination during filling or drying. A filling lot must
therefore have been filled in one working session and (if applicable)
have been dried together in the same chamber.
Tissue culture infective dose 50% (TCID<sub>50</sub>): the quantity of a virus suspension that will infect 50% of cell cultures.

Mouse lethal dose 50% (mouse LD<sub>50</sub>): the quantity of a virus suspension that will kill 50% of mice injected with it.

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) (4, p. 11) shall apply to establishments manufacturing rabies vaccine for veterinary use.

The production of rabies vaccine shall be conducted by staff who have not handled other infectious microorganisms or animals in the same working day. The staff shall consist of persons who shall be examined medically and found to be healthy. Steps shall be taken to ensure that all such persons in the production and control areas have been immunized against rabies and have an antibody titre of at least 0.5 IU per ml of serum.

Only the cultures approved by the national control authority for the production of rabies vaccine shall be introduced or handled in the production area.

Persons not directly concerned with the production processes, other than official representatives of the control authority, shall not be permitted to enter the production area.

Particular attention shall be given to the recommendations contained in Part A, section 1, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) 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 as formulated in the requirements of Part A, section 3 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Estab-
lishments and Control Laboratories) (4, p. 15) shall apply to the manufacture of rabies vaccine.

3.1 Control of source materials

3.1.1 Strain of virus

The strain of virus used in the production of all seed lots shall be a “fixed” strain and shall be identified by historical records. It shall have been shown, to the satisfaction of the national control authority, to yield a safe and immunogenic vaccine which stimulates immunity lasting for at least one year in all the species for which the vaccine is intended. In addition, the vaccine strain shall be characterized by serological tests (e.g., immunofluorescence) and animal inoculation.

Records shall be maintained of all tests for verification of strain characters to be made each time the working seed is changed. Such tests shall include the titration in animals of various species and ages and by various routes of inoculation as well as serum neutralization tests. The tests shall be those approved by the national control authority.

For inactivated vaccines the most common production strains originate from the Pasteur strain of rabies “fixed” virus, maintained historically in rabbit passage, or a derivative of such a strain.1 Such strains should be capable of producing characteristic paralysis within 5–7 days when inoculated intracerebrally into rabbits.

3.1.2 Substrates for the production of seed virus and vaccine

3.1.2.1 Animals. Only healthy animals shall be used. They shall conform to the requirements given in Part A, section 3.2.1.

Different species of animals may be used for vaccine production or for preparing seed virus. Rabbits (adult or preferably suckling), sheep, goats, suckling mice, and suckling rats are used in different countries.

3.1.2.2 Embryonated eggs. Only embryonated eggs obtained from healthy flocks shall be used.

Tests to demonstrate freedom from avian pathogens may be required in some countries.

1 This strain is available to laboratories, on request and with the approval of the national authorities, from the World Health Organization, Geneva, Switzerland.
In some countries the whole group of birds is bled on the establishment of the flock, and thereafter a 5% sample of the birds is bled each month. The serum samples are screened for freedom from antibodies to chicken pathogens. Any bird that dies is investigated to determine the cause of death.

3.1.2.3 Cell cultures. Cell cultures used in the production of rabies seed virus or vaccine shall be approved by the national control authority.

Cell cultures shall be made from tissues derived from healthy animals.

All information on the source and method of preparation of the cell culture system used shall be available to the national control authority. Where applicable, details of any prophylactic and diagnostic measures to which the animals serving as a source of tissue may have been subjected and data showing freedom from infectious agents by the monitoring of antibody production shall be provided.

If chick embryo tissue is used for the propagation of rabies virus, the eggs used as a source of tissue shall be derived from healthy flocks.

In some countries flocks are required to be free from Salmonella infection, Mycobacterium avium, mycoplasma, and other agents pathogenic for chickens.

If dog kidney tissue is used for the propagation of rabies virus, it shall be obtained either from dogs that are in overt good health and have been maintained in quarantine in vermin-proof quarters for a minimum of six weeks, having had no exposure to nonquarantined dogs or other animals throughout the quarantine period, or from dogs born in quarantine, provided that they have been kept in the same type of quarantine continuously from birth.

In some countries the dogs are kept in quarantine for six months.

Each dog should be examined periodically during the quarantine period and, at the time of use, autopsied by a pathologist qualified in, or a physician or veterinarian having experience with, diseases of dogs, for signs of disease, particularly tuberculosis, infectious canine hepatitis, canine distemper, rabies, leptospirosis, and other diseases of dogs. If any such signs or significant pathological lesions are observed, tissue from such animals should not be used.

If fetal bovine kidney tissue is used for the propagation of rabies virus it shall be obtained from the fetuses of cows that are in overt good health and have passed pre- and postmortem examinations.
Kidney tissue should be obtained from fetuses of cows that have not been used previously for purposes involving infectious agents. If any sign of illness or significant pathological lesions are observed in pregnant cows the fetal tissue from such animals should not be used.

Special attention should be given to foot and mouth disease, brucellosis, Q fever, leptospirosis, rinderpest, tuberculosis, and bovine virus diarrhoea, but in some areas other diseases of cattle may also have to be considered.

If hamster kidney tissue is used for the propagation of rabies virus, only hamsters of a strain approved by the national control authority shall be used as a source of tissue.

The animal stock should be free from infection with mycoplasma, and from microorganisms pathogenic for hamsters. The parents of animals to be used as a source of tissue should be maintained in quarantine in vermin-proof quarters for a minimum of three months. Neither the parent hamsters nor their progeny should previously have been used for experimental purposes involving infectious agents.

If diploid cells or continuous cell lines are used for the propagation of rabies virus they shall be those approved by and registered with the national control authority. The cells shall have been characterized with respect to their genealogy, growth characteristics, viability during storage, and karyology, and they shall have been shown, by tests in animals, eggs and cell cultures, to be free from detectable adventitious agents.

3.1.2.4 Serum used in cell culture medium. Serum used for the propagation of cells shall be tested to demonstrate freedom from bacteria and fungi according to the requirements given in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (5, p. 49), and freedom from mycoplasma and viruses if required by the national control authority.

3.1.3 Virus seed lot system

The preparation of rabies vaccine shall be based on the use of a virus seed lot system. A working seed lot shall be not more than 10 passages removed from the master seed lot, which has been thoroughly characterized. Vaccines shall be made from a working seed lot without further intervening passage. Seed lots shall be maintained either
in the dried or in the frozen form and be stored separately. If frozen, the seed shall be kept continuously at a temperature below \(-60^\circ\text{C}\). Seed lots shall have been shown, to the satisfaction of the national control authority, to be capable of yielding vaccine that meets all these requirements.

### 3.1.4 Tests on virus seed lots

Each seed lot shall be identified as rabies virus by methods approved by the national control authority. An immunofluorescence test is suitable.

**3.1.4.1 Freedom from bacteria, fungi, and mycoplasmas.** Each seed lot shall be tested for bacterial, mycotic, and mycoplasmal contamination by appropriate tests according to the requirements of Part A, section 5.2 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (5, p. 49).

**3.1.4.2 Virus content.** A titration of virus content of each seed lot shall be made.

Such titrations may be done by the intracerebral inoculation of suitable dilutions in mice or in cell cultures. The mice are observed for at least 14 days.

**3.1.4.3 Freedom from extraneous agents.** In addition to the tests described in Part A, section 3.1.4.1, each seed lot used for the production of vaccine shall be tested for extraneous agents in animals and cell cultures after neutralization of the virus with a monospecific antirabies serum.

The individual tests on the seed virus shall depend on its passage history and the nature of the substrate used for the growth of the virus and shall be approved by the national control authority.

### 3.2 Production of vaccine

Antibiotics shall not be added to the final vaccine.

If antibiotics are used during the production of the vaccine made in chick embryos or cell cultures the quantity allowed in the final product should be determined by the national control authority. In some countries the addition of antibiotics during manufacture is not allowed.
3.2.1 Vaccines produced in neural tissue

The animals intended for production shall be kept in quarantine under veterinary supervision for at least two weeks prior to inoculation of the seed virus, except in the case of suckling animals, when this requirement shall apply to the mothers. Only animals free from all signs of disease shall be used. Seed virus shall be inoculated intracerebrally. Methods for inoculation and harvesting approved by the national control authority shall be used.

While virus is always inoculated intracerebrally, the technique used varies with the species of animal. A satisfactory technique is one that consistently produces paralysis in the inoculated animals but does not introduce other infection.

In order to obtain the maximum virus titre, neural tissues from inoculated animals, apart from suckling animals, should be harvested on an individual basis, when the animal shows signs of advanced rabies.

If suckling animals are used, the dose and date of inoculation should allow for harvesting of neural tissue before neuroallergenic activity becomes demonstrable. This can be done for the animal species and particular strain used for vaccine production by immunizing guinea-pigs with neural material suspended in complete Freund adjuvant. It is essential that positive and negative controls should be included in the test. On the basis of the results of the test, the period can be assessed during which acceptable material can be harvested. The time of harvest used by some production laboratories is 8 days for mice, 6 days for rabbits, and 7–11 days for rats.

Neural tissue shall not be taken from dead animals, whether death is due to rabies or to other causes.

All animals used in the production of vaccine should be examined by autopsy after the removal of neural tissue. If evidence of tuberculosis or any neural disease other than rabies is found, the nerve tissue from the animal should be discarded, or if nerve tissues have been pooled, the pool containing nerve tissue from such an animal should be discarded. If there is evidence of a communicable disease among the animals, the viral harvest from that group should be discarded.

If sheep are selected for vaccine production, histological examination of the brain should be included in the postmortem examination to exclude the possibility of listeriosis.

When other than suckling animals are used, the tissue harvested from each animal shall be kept separate until completion of the sterility test (Part A, section 3.3.1). When suckling animals are used, the harvest composed of a pool of tissue from a group of animals inocu-
lated at the same time and harvested together shall similarly be kept separate until completion of the sterility test.

3.2.2 Vaccines produced in embryonated eggs

The eggs shall be derived from healthy flocks free from microorganisms known to be pathogenic for poultry.

Such agents include *Salmonella pullorum*, *Mycobacterium tuberculosis*, avian mycoplasma, and avian leucosis viruses.

After the eggs have been incubated for a suitable period they shall be inoculated with seed virus. After further incubation for a suitable period, the living embryos shall be harvested with aseptic precautions. Embryos inoculated at the same time and harvested together may be pooled and the viral harvest kept separate until completion of the sterility test (Part A, section 3.3.1).

3.2.3 Vaccines produced in cell cultures

At least 5% of the cell suspension (not less than 500 ml and not more than 1 litre) at the concentration employed for seeding vaccine production cultures shall be used to prepare control cultures.

In some countries in which the technology of large-scale production has been developed, the national control authority should determine the size of the cell sample to be examined. Where a cell bank has been established and tested for freedom from extraneous agents, the national control authority may amend the tests on the control cells.

The treatment of cells set aside as control material shall be similar to that of the production cell cultures, but they shall remain uninoculated as control cultures for the detection of extraneous viruses.

These control cell cultures shall be incubated under the same conditions as the inoculated cultures for at least two weeks or until the time of the last harvest of the production cultures—whichever is the later—and shall be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures shall have had to be discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures shall be examined for degeneration caused by an infective agent. If this examination, or any of the tests specified in this section, shows evi-
dence of the presence in a control culture of any adventitious agent, the rabies virus grown in the corresponding inoculated cultures shall not be used for vaccine production.

3.2.3.1 *Tests for haemadsorbing viruses.* At the end of the observation period, cells comprising 25% of the control cells shall be tested for the presence of haemadsorbing viruses, using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage shall not have exceeded 7 days, and the temperature of storage shall have been in the range of 2–8°C.

In some countries the national control authority requires that tests for haemadsorbing viruses should be made in addition, using other types of red cells including those from humans (blood group IV, O) and chickens (or other avian species). The cultures should be examined at 3–5 days and again at 12 days. All tests should be read after incubation for 30 min at 0–4°C and again after a further incubation for 30 min at 20–25°C.

3.2.3.2 *Tests for other extraneous agents.* Fourteen days after the day of initiating the control cells or at the time of the last harvest—whichever is the later—a sample of the pooled fluids shall be taken at each period of collection from each group of control cultures. Ten ml of each pool shall be tested in the same cells, but not the same batch of cells as that used for the production of virus growth.

The inoculated cultures shall be incubated at a temperature of 35–37°C and shall be observed for a period of at least 14 days.

Some national control authorities require that at the end of this observation period a subculture shall be made in the same cell culture system and observed for at least 7 days. Furthermore, some national control authorities require that these cells should be tested for the presence of haemadsorbing viruses.

For the tests to be valid, at least 80% of the culture vessels shall be available and suitable for evaluation at the end of the respective test periods.

If any cytopathic changes occur in any of the cultures, the virus harvests produced from the batch of cells from which the control cells were taken shall be discarded.

3.2.3.3 *Additional tests on control cells if avian embryo cells are used for production.* A sample of the control fluid taken at the end of the observation period of the control cell cultures shall be tested for avian leucosis viruses and adenoviruses.
In some countries the complement fixing test (COFAL) is used for detecting avian leucosis viruses, and liver or kidney cell cultures of embryos are used for detecting adenoviruses.

3.2.3.4 Additional tests on control cells if other cell cultures are used. When other cell cultures are used for the growth of rabies virus, additional tests for the detection of viruses specific for the host species shall be included. Such tests shall, to the satisfaction of the national control authority, be suitable for the purpose.

Only those cells shown to be free from contamination shall be used.

3.3 Control of bulk material

3.3.1 Sterility tests of the viral harvest

A sample removed from each viral harvest shall be tested for bacterial and fungal contamination by appropriate tests according to the requirements of Part A, section 5.2 of the revised Requirements for Biological Substances, No. 6 (General Requirements for the Sterility of Biological Substances) (5, p. 48). Any viral harvest in which contamination is detected shall be discarded.

3.3.2 Pooling of viral harvests

Only viral harvests satisfying the requirements for sterility given in Part A, section 3.3.1 of these requirements shall be pooled for bulk material.

In some countries the viral harvests are tested for the presence of extraneous viruses after neutralization of the rabies virus. The tests should be approved by the national control authority.

Bulk material shall not be subjected to repeated freezing and thawing. A sample shall be taken from the homogenized bulk material prior to inactivation for determination of the virus titre in mice or on cell cultures.

3.3.3 Homogenization and virus titration

The apparatus used for homogenizing the brain tissue and embryos shall be of such a design as to prevent any escape of aerosols. The apparatus should be housed in a safety cabinet.

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The grinding and blending of tissues should be done at as low a temperature as possible to avoid destruction of virus.

Neural tissue vaccines should be prepared in such a way that a single dose is contained in not more than 2 ml of a 5% nerve tissue suspension or its equivalent—e.g., 1 ml of a 10% suspension.

3.3.4 Inactivation procedure

Methods and agents used for inactivation shall be approved by the national control authority. For vaccines made in neural tissue, inactivation shall be initiated immediately after homogenization. For vaccines made in cell cultures, inactivation shall be commenced immediately after clarification or filtration. The method used shall be demonstrated to be consistently effective in the hands of the manufacturer. For vaccines made in neural tissue the inactivation process shall also have been shown, to the satisfaction of the national control authority, to be capable of inactivating mycoplasma, as demonstrated by in vitro tests. In the case of vaccine produced in the brain of suckling animals, the method shall be shown to inactivate any extraneous agent that may be present, as demonstrated by tests using tissue culture or by animal inoculation. The inactivation of the virus must be completed within one half of the total inactivation time.

Various methods for inactivating rabies virus have been used with success. The concentration of the inactivating agent, the temperature, and the length of time necessary for inactivation must be established for the particular type of vaccine being manufactured. A widely used agent is phenol, generally at a concentration ranging from 0.5% to 1% and at a temperature of 20–30°C for several days until complete inactivation has occurred, as demonstrated by the results of the test specified in Part A, section 3.3.6. Etherized vaccines are produced in some countries by combining the action of ether and phenol in the inactivation procedure. Beta-propriolactone (BPL) is also used. Satisfactory vaccines may be prepared by treating 10% neural tissue homogenates at 20°C with a concentration of 1:3500 to 1:5000 BPL for 24 h or until complete inactivation has occurred, as demonstrated by the results of the test specified in Part A, section 3.3.6.

Ultraviolet light irradiation has also been used, but the equipment required and the procedures involved make it difficult to prepare vaccine in large volumes. The dosage range and time of application needed to accomplish complete inactivation of the virus without reducing antigenicity are critical, but when the radiation dose is regulated properly, highly antigenic vaccines may be prepared. The time required for inactivation is short compared with that needed when chemical methods are used,
and hence the vaccine may be kept at a low temperature throughout; this also aids in conserving antigenicity.

Vaccines may be freeze-dried. For the best results the lapse of time from inactivation to initiation of the freeze-drying cycle should be kept to a minimum.

3.3.5 Concentration and purification of virus harvest

If concentration and purification of the virus harvest are carried out, the methods used shall be approved by the national control authority. The manufacturer shall show, to the satisfaction of the national control authority, that concentration has been effected.

The potency test is a useful measure of the degree of concentration achieved.

3.3.6 Test for effective inactivation

For inactivated vaccines intracerebral inoculation of mice shall be used to test each bulk material for inactivation of virus prior to the addition of preservatives and other substances. The tests shall be approved by the national control authority.

The test should be performed with undiluted bulk material injected intracerebrally into at least 20 mice, each weighing between 15 and 20 g. In some countries similar tests are required in 4-day-old mice or in rabbits or guinea-pigs.

In some cases the concentration of inactivating agent or tissue in undiluted bulk material may be toxic to the test animals. In this case the test should be performed on final bulk material, which may be diluted, if necessary, but the dilution should not be more than 1:2.

In some countries 2 species of animal are used—e.g., rabbits and mice—for testing effective inactivation. In such cases at least 3 rabbits should be used. If the virus was propagated in an animal other than the rabbit, consideration should be given to using the production species rather than the rabbit.

For vaccine produced in cell cultures the rabies virus amplification test shall be used in the cell culture of origin to test for the presence of live virus. At least 25 ml of virus pool shall be tested in this way (6) or by another method of similar sensitivity.

The bulk material passes the test if the product has been shown, to the satisfaction of the national control authority, to be free from residual live virus.
3.4 Preparation and control of final bulk

3.4.1 Preservatives and other substances added

In preparing the final bulk, only the preservatives or other substances 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 amounts used.

If phenol has been used for inactivation, its concentration in the final bulk shall be such that it will not exceed 0.5% in the final product.

If beta-propiolactone has been used for inactivation, the procedure shall be such that there is no detectable amount of the chemical in the final bulk.

A suitable adjuvant may be added. The type and concentration of the adjuvant shall be approved by the national control authority.

No antibiotics shall be added to rabies vaccine.

3.4.2 Potency test on the final bulk

The manufacturers may wish to carry out a potency test on the final bulk. If this test is done, however, it will not eliminate the need for the test for potency that must be performed on the final product.

For inactivated vaccine made in cell cultures a suitable test to be applied to the bulk is the antibody binding test (7).

3.4.3 Sterility tests

Each final bulk shall be tested for sterility according to the requirements given in Part A, section 5.2 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (5, p. 48).

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) (4, p. 16) shall apply, with the addition of the following directives:

Containers of dried vaccine shall be hermetically sealed under vacuum or after filling with pure, dry, oxygen-free nitrogen or any
other gas not deleterious to the vaccine. All containers shall be tested for leaks and all defective containers shall be discarded.

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 an appropriate method.

The test for potency as described in Part A, section 5.4 may serve as an identity test.

5.2 Sterility tests

Each filling lot shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5.2 of the revised Requirements for Biological Substances (General Requirements for the Sterility of Biological substances) (5, p. 48).

For the live virus vaccines, each filling lot shall also be tested for the presence of mycoplasma (5, p. 52).

5.3 Innocuity tests

5.3.1 Abnormal toxicity test

Each filling lot shall be tested for abnormal toxicity by appropriate tests in mice and guinea-pigs, using parenteral injections. The test procedures shall be those approved by the national control authority.

5.3.2 Safety tests

(1) Inactivated vaccines: Inject each of not less than two healthy susceptible animals of each of the species in which the vaccine is intended to be used, by the route stated on the label, with twice the vaccinating dose and observe for not less than 21 days. No abnormal local or systemic reaction shall develop.

(2) Live vaccines: Inject each of not less than two healthy non-vaccinated animals of each of the species in which the vaccine is intended to be used, by the route stated on the label, with 10 times the vaccinating dose and observe for not less than 28 days. No abnormal local or systemic reaction shall develop.
5.4 Potency tests

5.4.1 Potency test of inactivated vaccines

A test for potency shall be made on each filling lot. Before the test is made, dried vaccine shall be reconstituted to the form in which it is to be used.

The test shall be one in which mice are immunized and subsequently challenged with rabies virus and shall be made in parallel with a reference vaccine (8). The challenge strain and reference vaccine as well as the test procedure used shall be those approved by the national control authority (see Part B, section 1).

The potency of each filling lot shall not be inferior to that of vaccines which have been shown to be efficacious in all species of animal for which the vaccine is recommended. The minimum potency shall be approved by the national control authority.

In some countries it is required that rabies vaccines for veterinary use shall have a potency of at least 0.3 IU. This has been shown to be a satisfactory guarantee for immunogenicity of the vaccines. In view of the progress made in the field of veterinary vaccines it seems advisable to change the minimal relative potency to 0.8 IU.

Reproducibility of the test depends in part on the strain of rabies virus used for challenge and its maintenance in a large homogeneous working pool kept below −60°C (preferably after centrifugation). The strain of mice may also affect reproducibility.

When the NIH test is used, the potency relative to a reference preparation is determined (8). It is preferable to use 7 vaccine dilutions with a logarithmic dilution ratio of 0.7 with 10 mice per dilution.

A reference preparation with an activity calibrated in international units by comparison with the third International Reference Preparation of Rabies Vaccine is included in each test. The potency of the vaccine in international units is then determined by comparing its activity with that of the reference preparation.

5.4.2 Potency test of live vaccines

For live vaccines, a test for potency shall be made on each lot. The test shall be one in which the amount of infective rabies virus is determined. The method shall be approved by the national control

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* A suitable challenge strain, CVS, is available to laboratories, on request and with the approval of the national authorities, from Veterinary Public Health, World Health Organization, Geneva, Switzerland.
authority and have been previously used to establish the amount of infective virus necessary to be effective in host animals. The titre of each filling lot shall be not less than that proved as efficacious in all species of animals for which the vaccine is recommended. The minimum virus titre shall be approved by the national control authority.

In most countries, titrations are done by the intracerebral inoculation of serial dilutions into young adult mice or, for some strains, suckling mice. Infective virus may also be quantified by using a plaque assay or fluorescent assay in tissue culture.

5.5 Stability test for freeze-dried vaccine

The method of production of vaccine shall be such that stable vaccine is produced as shown by an accelerated degradation test. The test used shall be approved by the national control authority.

The test for potency (see Part A, section 5.4), made after the storage of samples for 2 weeks at 37 °C for the inactivated vaccine and for 1 week at 37 °C for the live vaccine, is suitable. In order to pass the test the vaccine should retain the minimum potency, as defined in Part A, section 5.4.1.

In some countries each lot of vaccine must be subjected to the stability test, whereas in others the test is required only for initial licensing lots to show consistency of production.

5.6 Residual moisture test on freeze-dried vaccine

In the case of dried vaccine it is advisable to test for residual moisture, as a guide to the maximum content allowable for the stability of the product. With some vaccines it is possible to dry the product to less than 1% residual moisture without impairing its stability and potency. However, depending on the type of stabilizer present, higher values may be accepted by the national control authority.

5.7 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) (4, p. 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) (4, p. 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) (4, p. 18) shall apply, with the addition of the following directive:

The leaflet accompanying the package shall include the following information:
— the tissue and animal species in which the vaccine was prepared;
— the method used for inactivating the virus;
— if the vaccine is in the dried form, a statement that, after its reconstitution it shall be used as soon as possible or stored at 5 ± 3°C and discarded at the end of the day;
— the route of administration of the vaccine.

In some countries it is required that all rabies vaccines should be given by the intramuscular route. Vaccines with an adjuvant are often administered subcutaneously.

9. DISTRIBUTION AND SHIPPING

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) (4, p. 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 Require-
ments for Manufacturing Establishments and Control Laboratories) (4, p. 19) shall apply.

10.1 Storage conditions

Rabies vaccine shall be stored at a temperature of 5 ± 3 °C.

10.2 Expiry date

The expiry date shall be based on data submitted by the manufacturer and shall be determined by the national control authority.

It has been usual to allow an expiry date of 12 months for the liquid vaccine and 18 months for the dried vaccine, the period concerned starting from the last satisfactory potency assay.

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) (4, p. 19) shall apply.

The national control authority shall give directions to manufacturers concerning the strain of rabies virus\(^3\) to use for production of vaccine.

The national control authority shall provide or approve the strain for challenge\(^3\) and the reference vaccine for use in the potency test (Part A, section 5.4).

2. RELEASE AND CERTIFICATION

A vaccine lot shall be released only if it fulfils Part A of these requirements.

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\(^3\) The Pasteur strain for vaccine production and the CVS virus are available to laboratories, on request and with the approval of the national authorities, from Veterinary Public Health, World Health Organization, Geneva, Switzerland.
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 whether or not the lot of vaccine in question meets all national requirements as well as Part A of the present requirements. The certificate shall further 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 rabies vaccine (for veterinary use) between countries.

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ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice and for supplying additional data relevant to these Requirements:

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REFERENCES

Appendix 1

SUMMARY PROTOCOL FOR RABIES VACCINE
FOR VETERINARY USE—LIVE
PRODUCTION AND TESTING

Identification of Final Lot

Name and address of manufacturer

Lot number

Date of manufacture of final lot

Expiry date

Type of vaccine (embryonated egg/cell
culture)

Animal species for which vaccine is
intended

Vaccine Virus Strain

Designation and short description of
origin and history

Designation and date of establishment of
master seed virus

Designation and date of establishment of
working seed

Number of passages between master and
working seed

Virus Production Substrate

(Complete only relevant part A, B, or C.)

A. Embryonated eggs

Animal species

Origin of eggs

Pre-inoculation period

122
### B. Cell cultures/primary

<table>
<thead>
<tr>
<th>Description</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of cells (kidney, fetal kidney, etc.)</td>
<td></td>
</tr>
<tr>
<td>Animal species of cells</td>
<td></td>
</tr>
<tr>
<td>Controls performed on animals from which the cells originated</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
</tr>
<tr>
<td>Amount of cell culture inoculated</td>
<td></td>
</tr>
<tr>
<td>Amount of control cell culture investigated</td>
<td></td>
</tr>
<tr>
<td>Tests performed on control cultures</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
</tr>
</tbody>
</table>

### C. Cell cultures/diploid or continuous

<table>
<thead>
<tr>
<th>Description</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin and short history of cell seed</td>
<td></td>
</tr>
<tr>
<td>Authority by which cell seed was approved</td>
<td></td>
</tr>
<tr>
<td>Amount of cell culture inoculated</td>
<td></td>
</tr>
<tr>
<td>Amount of control cell culture investigated</td>
<td></td>
</tr>
<tr>
<td>Tests performed on control cultures</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
</tr>
</tbody>
</table>

---

### Serum for Cell Cultures

<table>
<thead>
<tr>
<th>Description</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin of serum used</td>
<td></td>
</tr>
<tr>
<td>Tests performed on serum</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
</tr>
</tbody>
</table>
Bulk Material

Have all the harvests included been tested for sterility?

Result of sterility tests on each viral harvest

Number of viral harvest pooled

Nature of concentration and/or purification (if applied)

Virus titre

Final Bulk

Preservatives, etc.

Concentration of antibiotics

Stabilizer used

Result of sterility test on final bulk

Test on Final Lot

1. Identity test

   Method used

   Result

2. Sterility tests

   Number of containers examined

   Method of test

   Date at start of test

   Date at end of test

   Result

3. Innocuity test

   Abnormal toxicity

   Mice

   Guinea-pigs

   Number of animals

   Route of inoculation

124
Volume of inoculation
Date of inoculation
Date at end of test
Result

Safety
Animal species used
Number of animals
Volume and route of inoculation
Date of inoculation
Date at end of test
Result

4. Potency test
Method used
Date of inoculation of vaccine
Minimum titre required for animals for which the vaccine is recommended
Titre found

5. Stability test for freeze-dried vaccine
Duration and temperature of incubation
Result

6. Residual moisture test for freeze-dried vaccine
Method used
Result

Signature of head of laboratory

125
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 Rabies Vaccine for Veterinary Use.

Date ____________________________________________

Signature _______________________________________

Name typed ______________________________________

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

Appendix 2

SUMMARY PROTOCOL FOR RABIES VACCINE FOR VETERINARY USE—INACTIVATED PRODUCTION AND TESTING

Identification of Final Lot

Name and address of manufacturer ______________________________________

Lot number ______________________________________

Date of manufacture of final lot ______________________________________

Expiry date ______________________________________

Type of vaccine (neural tissue/cell culture) ________________________________

Animal species for which vaccine is intended ______________________________

Vaccine Virus Strain

Designation and short description of origin and history _____________________

Designation and date of establishment of master seed virus ________________

126
<table>
<thead>
<tr>
<th><strong>Designation and date of establishment of working seed</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of passages between master and seed</strong></td>
</tr>
</tbody>
</table>

**Virus Production Substrate**

(Complete only relevant part—i.e., A, B, or C.)

**A. Animal neural tissue (brains)**

<table>
<thead>
<tr>
<th>Animal species</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Quarantine period</td>
<td></td>
</tr>
<tr>
<td>Adult/suckling</td>
<td></td>
</tr>
<tr>
<td>Number of animals used</td>
<td></td>
</tr>
<tr>
<td>Age of animals</td>
<td></td>
</tr>
<tr>
<td>Period between inoculation and harvest</td>
<td></td>
</tr>
<tr>
<td>Result of autopsy</td>
<td></td>
</tr>
</tbody>
</table>

**B. Cell cultures/primary**

| Type of cells (kidney, fetal kidney, etc.) |                          |
| Animal species of cells |                                    |
| Controls performed on animals from which the cells originated |                                |
| Result |                                    |
| Amount of cell culture inoculated |                                    |
| Amount of control cell culture investigated |                          |
| Test performed on control cultures |                                    |
| Result |                                    |

**C. Cell cultures/diploid or continuous**

| Origin and short history of cell seed |                          |
| Authority by which cell seed was approved |                                    |
| Amount of cell culture inoculated |                                    |
Amount of control cell culture investigated
Tests performed on control cultures
Result

Serum for Cell Cultures
Origin of serum used
Tests performed on serum
Result

Bulk Material
Result of sterility tests on each viral harvest
Number of viral harvest pooled
Nature of concentration and/or purification (if applied)
Virus titre
Method of inactivation
Temperature and duration
Test for inactivation material injected (including concentration)
Number of mice injected
Weight of mice
Other animals (if done)
Result of tests
Result of virus amplification test (for cell culture vaccines)
Volume of material tested

Final Bulk
Concentration of phenol (if used)
Concentration of antibiotics used
Other preservatives (type and concentration)
Adjuvant (if used)
Result of sterility test
1. **Identity test**

   Method used
   
   Result

2. **Sterility tests**

   Number of containers examined
   
   Method of test
   
   Date at start of test
   
   Date at end of test
   
   Result

3. **Innocuity tests**

   **Abnormal toxicity**

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Guinea-Pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Route of inoculation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume of inoculation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date of inoculation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date at end of test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

   **Safety**

   |                |      |             |
   | Animal species used |    |             |
   | Number of animals |      |             |
   | Route of inoculation |   |             |
   | Volume of inoculation | |             |
   | Date of inoculation |    |             |
   | Date at end of test |    |             |
   | Result              |      |             |

4. **Potency test**

   Type of test

   ____________________________

129
Immunization of mice

Date at start of test
Reference vaccine (potency)
Challenge strain
Date of challenge
ED₅₀ test vaccine
ED₅₀ reference vaccine
Calculated IU/dose
Confidence limits

5. Stability test for freeze-dried vaccine

Duration and temperature of incubation
Result

6. Residual moisture test for freeze-dried vaccine

Method used
Result

Signature of head of laboratory

Certification by person taking overall responsibility for production of the vaccine.
I certify that lot No. [lot number] of the vaccine satisfies Part A of the WHO Requirements for Rabies Vaccine for Veterinary Use.

Date
Signature
Name typed

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

Many viruses may infect the liver of animals and man and may produce severe diseases. The general term “viral hepatitis” refers to infection caused by hepatitis virus type A or type B, which can now be differentiated by specific laboratory tests for antigens and antibodies associated with these infections, and by a more recently identified and unrelated type of hepatitis referred to as “non-A, non-B hepatitis”. This new form of hepatitis may be caused by more than one agent.
Viral hepatitis is a major public health problem occurring endemically in all parts of the world. Acute viral hepatitis is a systemic or generalized infection with the liver as the target organ. The clinical picture therefore includes inapparent or subclinical infection, mild gastrointestinal symptoms of the anicteric form of the disease, acute illness with jaundice, severe prolonged jaundice, and acute fulminant hepatitis.

Hepatitis A is usually spread by person-to-person contact by the faecal-oral route, and major outbreaks result most frequently from faecal contamination of water and food. The virus, which has been identified as an RNA virus with the characteristics of members of the *Enterovirus* genus, has recently been cultivated in tissue culture and progress towards development of a vaccine is expected.

There is substantial evidence that hepatitis B may also progress to chronic liver disease, including chronic persistent hepatitis, chronic active (aggressive) hepatitis, and cirrhosis, and there is evidence of an association between hepatitis B and primary hepatocellular carcinoma.

Hepatitis B virus (HBV) has been identified as a 42-nm particle (known as Dane particle) containing double-stranded DNA. Infection with hepatitis B virus is manifested by at least three antigenic components: hepatitis B surface antigen (HBsAg), the core antigen, and the e antigen(s) (HBeAg), resulting from replication of the virus in the hepatocytes. The surface antigen is most frequently found as 20–22-nm spherical particles (sometimes slightly larger or smaller) and tubular forms, and possesses a common determinant a and generally at least two mutually exclusive subdeterminants d or y and w or r. Other subspecificities have also been recognized.

Hepatitis B virus has not yet been cultivated in cell culture and small laboratory animals are not susceptible to infection. The infection can be transmitted to certain of the apes, of which the chimpanzee is the only available susceptible animal model for hepatitis B and both short- and long-incubation forms of non-A, non-B hepatitis.

The importance of hepatitis B may also be considered under a variety of headings, which include its effects on every field of medical practice, the impact that it has on blood transfusion services, and its association with progression to chronic liver disease. In addition, infection with hepatitis B may be followed by the persistent carrier state. Such a carrier state may be associated with liver damage. It is estimated that the number of carriers in the world amounts to about 176 million, with approximately the following geographical distribution, particularly among blood donors: northern Europe, North America
and parts of Australia, a prevalence of 0.1% or less; a prevalence of up to 5% in central and eastern Europe, with a higher frequency in southern Europe and countries bordering the Mediterranean; a similar frequency of 5% or more in parts of Central and South America; and a prevalence of up to 20% in some parts of Africa, Asia, and the Pacific area.

The importance of the parenteral and inapparent parenteral routes of transmission of hepatitis B virus is now well established, and although various body fluids, such as saliva, menstrual and vaginal discharges, seminal fluid, breast milk, and serous exudates, have been considered in the spread of infection, infectivity appears to be especially related to blood. Transmission of hepatitis B infection from carrier mothers to their babies can occur during the perinatal period and appears to be an important factor determining the prevalence of the virus in some regions.

There is an urgent need for a hepatitis B vaccine, particularly for groups that are at increased risk of acquiring infection (1). Since it has been shown that the separated viral coat proteins, containing hepatitis B surface antigen, lead to the production of protective antibody, it is now possible to use purified and inactivated 22-nm spherical hepatitis B surface antigen particles, or subunits derived from the surface antigen, as vaccines. However, the preparation of such vaccines for use in man from human viral antigens not grown in cell culture, but obtained from the plasma of infected persons—namely, from persistent carriers of hepatitis B antigens—is an entirely new approach in preventive medicine and will demand special consideration in the tests applied to the production and quality control of the vaccines.

**GENERAL CONSIDERATIONS**

At present the virus of hepatitis B cannot be reliably propagated in the laboratory in vitro. For this reason the preparation of a vaccine from virus grown in cell cultures is not possible. The discovery of a so-called Australia antigen in the serum of an Australian aborigine and the subsequent demonstration that this antigen was in fact the surface antigen of hepatitis B virus in an infected carrier of hepatitis B opened the door to the means for detecting hepatitis B carriers, for laboratory diagnosis, and for vaccine development even in the absence of any means of propagating the virus in the laboratory.
In 1971, Krugman first utilized this knowledge of the relationship of the Australia antigen (now called hepatitis B surface antigen) to hepatitis B and prepared a "vaccine" for preventing the disease. This preparation consisted of boiled, diluted, infectious serum from a hepatitis B carrier. Though crude and nonstandardized and now known not to have been completely inactivated, the material did induce antibodies against hepatitis B surface antigen (anti-HBs) in human subjects and the inoculated subjects were protected against the disease when challenged.

These important developments provided the stimulus for studies in a number of laboratories to prepare inactivated HBsAg vaccines using HBsAg purified from plasma obtained from antgenaemic carriers of hepatitis B. Vaccines of varying degrees of purity and technological sophistication have been prepared in a number of laboratories and some have been tested in humans.

Whatever the procedure used, it is universally accepted that the vaccine must be safe and potent—i.e., free from demonstrable exogenous virus and other microbial agents and capable of eliciting antibody against the virus in animals and in man by the administration of a standardized dose of antigen. In considering safety, emphasis has been given to the need for rendering the HBsAg substantially free from contaminating host substances. Inactivation by formaldehyde has been considered a desirable step, since formaldehyde is known to inactivate hepatitis B virus particles that might remain in the HBsAg preparation. In addition, formaldehyde can inactivate a wide range of viruses and provides additional safety; however, other means of inactivation may eventually be shown to be equivalent or even superior. The vaccine can be standardized for its antigen content on the basis of a total protein dosage and the amount of HBsAg estimated serologically. The values obtained in the two assays must be consistent to provide an additional measure of the purity of the product.

"In process" tests to detect the presence of other viruses can be carried out by conventional procedures. At present, the only means of detecting hepatitis B virus, however, is by the inoculation of susceptible chimpanzees, which are then tested with markers for evidence of hepatitis B virus infection. At present, chimpanzees are available only in limited number and it is necessary that they should be used prudently and an effort made to reuse them, after testing, for the establishment of breeding colonies.

Hepatitis B vaccines prepared to date have been incorporated in an adjuvant such as alum and all are assayed for their ability to stimulate
anti-HBs in animals, including mice, monkeys, chimpanzees, and
guinea-pigs. An extinction assay performed in mice may prove es-
pecially useful for the quantitative measurement of vaccine potency.
Tests on some of the vaccines have also been carried out in man.
One highly purified formaldehyde-treated vaccine in alum with a
high potency has been extensively tested in human subjects. The vac-
cine has so far been found to be safe, gives good antibody responses
in nearly all subjects given two doses of vaccine one month apart, and
shows substantial boosting effect when a third dose is given six months
later. This vaccine is now undergoing further studies to measure its
protective efficacy in special high-risk groups, including homosexu-
als, staff and patients in renal dialysis units, inpatients with thalassaemia,
and persons whose families include hepatitis B carriers. Other vac-
cines are also undergoing evaluation in man.

Controlled studies in chimpanzees have shown the efficacy of
several such vaccines in preventing hepatitis following challenge with
human hepatitis B virus.

Cross-protection studies carried out in chimpanzees suggest to date
that subtypes are not of major importance in vaccine composition.
Pending confirmation of this, however, those preparing vaccines have
so far chosen to prepare them from the monovalent αy or αd antigens.

Hepatitis B polypeptide vaccines are under development. Further-
more, as there is some evidence that HBV particles have antigenic
sites not present on the small particles, vaccines containing viral com-
ponents other than HBsAg are also under development.

Although the foregoing has presented the case for the formulation of
requirements for hepatitis B vaccine, it is recognized that in a
number of particulars it is not possible to be precise about the ab-
solute values that are required for a suitable vaccine. The two most
important factors are the quantity of antigen in the vaccine to give
protection and the details of the tests for the detection of infectious
virus. Although this field is undergoing rapid development, there are
data available to guide control authorities in determining these factors.
Another important consideration is the method of inactivation and
here again it would be important for the manufacturer to prove
efficacy if a new and improved method of inactivation were used.
In spite of these important decisions being left to the national control
authorities, who may obtain assistance from those intimately involved
in the production and control of hepatitis B vaccine, it is considered
important to formulate requirements for such a vaccine in order to
help control authorities to ascertain that imported vaccines meet the
requirements for safety and efficacy. It must be appreciated that this vaccine may be potentially dangerous unless the method of inactivation has been shown to give safe vaccine consistently.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements, so that, if a health administration so desires, these parts as they appear may be included in 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 hepatitis B vaccine, it is recommended that a clause should be included permitting modifications of manufacturing requirements on the condition that it can 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 final vaccine is manufactured and/or used.

PART A.
MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be “Vaccinum hepatitidis B”. 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 hepatitidis B is a preparation of purified, inactivated hepatitis B viral components. The preparation shall satisfy all the requirements formulated below.

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When cell cultures become available that are acceptable to the national control authorities the virus propagated in these cultures may be preferred.

1.3 International reference preparation and international unit

Since no international standards or reference preparations of hepatitis B vaccine have yet been established, no requirements for potency based on such standards or preparations can be formulated until such preparations become available. National control authorities should provide a reference preparation of hepatitis B surface antigen for use in tests of antigen concentration (see Part A, section 5.6).

For the calibration of techniques used in the measurement of antibody responses to hepatitis B vaccines, an international reference preparation is available. This preparation is calibrated in terms of anti-HBs.

The International Reference Preparation of Hepatitis B Immunoglobulin (established in 1977) is dispensed in ampoules containing 50 IU of hepatitis B immunoglobulin (fractionated human plasma, freeze-dried). This preparation is in the custody of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands.

1.4 Terminology

The following definitions are given for the purposes of these requirements only:

Whole blood (sometimes referred to as blood): the blood collected in an anticoagulant solution.

Plasma: the liquid part of blood collected in a receptacle containing an anticoagulant.

HBV: hepatitis B virus. A 42-nm double-shelled virus, originally known as the Dane particle.

HBsAg: hepatitis B surface antigen. The hepatitis B antigen found on the surface of the virus and on the accompanying unattached spherical (22-nm) and tubular particles.

HBCAg: hepatitis B core antigen. The hepatitis B antigen found within the core of the virus.

HBeAg: the e antigen(s) closely associated with hepatitis B infection.

Anti-HBs: antibody to hepatitis B surface antigen.

Anti-HBc: antibody to hepatitis B core antigen.

Anti-HBe: antibodies to the e antigen(s).
Single donor plasma: plasma obtained from a single donation of whole blood or obtained by plasmapheresis.

Plasma pools: pools of single donation plasmas that have been shown to be satisfactory before pooling.

Purified, inactivated HBsAg batch: purified and inactivated HBsAg prepared from one or more plasma pools.

Final bulk: the finished biological material prepared from one or more batches of HBsAg present in the container from which the final containers are filled. The final bulk may be prepared from one or more purified, inactivated HBsAg batches.

Final lot: a collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling or preparation of the finished vaccine. A final lot must therefore consist of finished material filled in one working session from a single final bulk.

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) (2, p. 11) shall apply to establishments manufacturing hepatitis B vaccine, with the addition of the following directives:

Production areas shall be decontaminated before they are used for the manufacture of hepatitis B vaccine.

The production of hepatitis B vaccine shall be conducted by staff who have not handled other infectious microorganisms or animals in the same working day. The staff shall consist of persons who shall be examined medically and found to be healthy.

In some countries steps are taken to ensure that all such persons in the production areas are immune to hepatitis B as shown by the presence of anti-HBs and are not hepatitis B carriers.

Persons not directly concerned with the production processes, other than official representatives of the national control authority, shall not be permitted to enter the production area.

Particular attention shall be given to the recommendations contained in Part A, section 1 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2, p. 13) regarding the
training and experience of 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 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) (2, p. 15) shall apply to the manufacture of hepatitis B vaccine.

3.1 The collection of blood and plasma

3.1.1 The selection of donors

Source materials for further processing are obtained from donations of blood or plasma. The medical criteria for accepting donors — criteria relating to the safety, purity, potency, and efficacy of the final products — must be the same for donors of whole blood (see Part A, section 3.1.2) components or blood components collected by plasmapheresis, except that the donors must be antigenemic and need not meet the exclusions relating to hepatitis.

In some countries separate areas or special times are set aside for the collection of plasma known to be contaminated with HBsAg.

3.1.2 Donors of whole blood

The physical fitness of a donor shall be determined by a licensed physician or a person under the direct supervision of a licensed physician. Donors shall be asymptomatic persons of either sex between the ages of 18 and 65 years, except that the findings in liver function tests may exceed normal limits.

3.1.3 Medical history

General. Before each donation questions shall be asked to determine that the donor is asymptomatic and has not suffered, or is not
suffering, from any serious illness—e.g., malignant disease, diabetes, epilepsy, hypertension, renal disease.

Any donor who appears to be suffering from symptoms of acute or chronic disease, or who is receiving oral or parenteral medication, with the exception of vitamins or oral contraceptives, may not be accepted for donation unless approved by a physician. The values obtained in liver function tests may exceed normal limits.

Any donor who appears to be under the influence of alcohol or any drug or who does not appear to be providing reliable answers to medical history questions shall not be accepted.

National health authorities shall develop policies designed to prevent the transmission of other infectious diseases based on the prevalence of these diseases in the donor population and the susceptibility of recipients to the same diseases.

**Minor surgery.** Donors shall not have a history of tooth extraction or other minor surgery during a period of 72 hours prior to donation.

**Pregnancy.** Pregnant women shall be excluded from blood donation. In general, mothers shall also be excluded for the period of lactation and for at least six months after full-term delivery.

**Immunization.** Symptom-free donors who have recently been immunized may be accepted with the following exceptions:

--- those receiving smallpox vaccine shall be excluded until the scab has fallen off or until two weeks after an immune reaction;
--- those receiving attenuated vaccines for measles (rubeola), mumps, yellow fever, or poliomyelitis shall be excluded until two weeks after the last immunization or injection;
--- those receiving attenuated rubella (German measles) vaccine shall be excluded until eight weeks after the last injection;
--- those receiving rabies (therapeutic) vaccine or immunoglobulin shall be excluded until one year after the last injection;
--- those receiving passive immunization using animal serum products shall be excluded until four weeks after the last injection.

3.1.4 **Physical examination**

Donors shall have a weight, blood pressure, pulse rate, and temperature within normal limits. Donors with any measurements outside the established normal limits of weight, blood pressure, and pulse rate may be accepted only if approved by the responsible licensed physician.
The following recommendations may be useful for guidance:

(1) Blood pressure. Systolic blood pressure between 12 and 24 kPa (90 and 180 mm Hg); diastolic blood pressure between 6.7 and 13.3 kPa (50 and 100 mm Hg).

(2) Pulse. Between 50 and 100 beats per minute and regular.

(3) Temperature. Oral temperature not exceeding 37.5 °C.

(4) Weight. Donors weighing less than 50 kg may be bled proportionately less than 450 ml per unit, provided all other donor requirements are met.

In some countries it is not required to take the body temperature but the decision to do so or not should be made by the national control authority.

Donors shall be free from any infectious skin disease at the venepuncture site and from skin punctures or scars indicative of addiction to narcotics.

### 3.1.5 Haemoglobin or haematocrit determination

The haemoglobin shall be not less than 125 g/l of blood for women and 135 g/l of blood for men; the haematocrit, if substituted, shall be not less than 38% or 41%, respectively.

These limits are not universally accepted, and national control authorities should raise or lower them when appropriate.

### 3.1.6 Donors for plasmapheresis

All phases of plasmapheresis, including explaining to donors what is involved in the process and obtaining their informed consent, shall be performed under the direct supervision of a licensed physician.

There are two groups of plasmapheresis donors: those who donate at a frequency comparable to that allowed for whole blood donations and those who donate more frequently. The former group shall be accepted on the basis of the above criteria for donors of whole blood.

In addition to these criteria, donors participating in the plasmapheresis programme shall be examined by a licensed physician on the day of the first donation, or no more than one week prior to the first donation. This examination shall include urine analysis and blood sampling for liver function tests, and determination of plasma proteins by electrophoresis or another suitable method.

On the day of each donation, in addition to meeting the requirements for whole blood donors, plasmapheresis donors shall be shown to have a total serum protein of no less than 60 g/l.
The medical evaluation of plasmapheresis donors shall be repeated at monthly intervals, as specified by national control authorities.

Whenever a laboratory value other than a liver function test is found to be outside the normal limits or any important abnormalities are noted in a donor's history or on physical examination, the donor shall be removed from the programme. The donor shall not return to the programme until the abnormal finding has returned to normal and the responsible physician has given approval.

If a plasmapheresis donor donates a unit of whole blood or does not have the red blood cells returned from a unit taken during the procedure, the donor shall be deferred for eight weeks unless special circumstances warrant approval by the responsible physician of earlier plasmapheresis. Plasmapheresis of donors of HBsAg-positive plasma will be permitted by the responsible physician, even if the liver function test values are above normal limits in individual donors.

Appropriate guidelines to define donor changes significant to justify discontinuation of plasmapheresis should be established by the responsible physician (3, p. 43).

The maximum volume of blood or plasma that may be taken in one year from chronic hepatitis B carriers shall be determined by the national control authority.

No guidelines have been established for the maximum volume of plasma that can be taken in any year from a chronic hepatitis B carrier.

In normal healthy subjects, some countries permit only 10 l to be collected whereas in one country as much as 60 l is allowed. Studies are needed to establish safe limits for the quantity of plasma to be collected from a chronic hepatitis B carrier. Such studies are under way.

3.2 Tests on single-donation plasma

Each single-donation plasma, whether obtained from whole blood or by plasmapheresis, shall be subjected to the following tests.

In some countries the tests are done on plasma pools, where this is approved by the national control authority.

3.2.1 Sterility tests

Single-donation plasma shall be tested for sterility according to the requirements given in Part A, section 5 of the revised Requirements.
3.2.2 Test for HBsAg

Each single donation shall be tested for the titre of HBsAg by a method approved by the national control authority.

Several tests are suitable for this purpose (7). Potency should be established with reference to a potency standard included in all assays.

3.2.3 Test for HBsAg subtype

In some countries, unless the subtype of a particular donor has been identified each single donation is tested for HBsAg subtype by a method approved by the National Control Authority. Several tests (among others, a gel diffusion test) have been shown to be suitable for this purpose (7).

3.3 Pooling of single-donation plasma

Only acceptable plasma shall be included in a plasma pool.

3.4 Tests on plasma pools

3.4.1 Sterility tests

A volume of at least 10 ml of each plasma pool shall be tested for sterility according to the requirements given in Part A, section 5 of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (4, p. 49).

3.4.2 Test for Mycobacterium tuberculosis

Each plasma pool shall be tested for the presence of mycobacteria (human) by culture methods approved by the national control authority.
3.4.3 Tests for extraneous viruses

3.4.3.1 Tests in animals and fertile eggs.

(1) Tests in adult mice

Each plasma pool shall be tested in adult mice for adventitious agents pathogenic to mice. Each of at least 10 adult mice, of 15–20 g weight, shall be inoculated intracerebrally with 0.03 ml and at least 10 mice intraperitoneally with at least 0.5 ml of the plasma pool. The mice shall be observed for at least 21 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined for evidence of viral infection, both macroscopically by direct 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 plasma pool passes 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 plasma pool.

(2) Tests in suckling mice

Each plasma pool shall be tested in suckling mice for adventitious agents pathogenic to mice. Each of at least 20 mice less than 24 hours old shall be inoculated intracerebrally with 0.01 ml and intraperitoneally with at least 0.1 ml of the plasma pool. The mice shall be observed daily for at least 14 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined to determine the cause of death or illness.

In some countries a subinoculation is suggested and in others an additional blind passage is made of a suspension of the pooled emulsified tissue (minus skin and viscera) of all mice surviving the original 14-day test.

The plasma pool passes 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 plasma.

(3) Tests in embryonated eggs

A sample of at least 5 ml of each plasma pool shall be tested in a group of embryonated hen 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 pool 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 plasma pool passes the test if there is no evidence of the presence of any adventitious agents attributable to the plasma pool.

3.4.3.2 *Tests in cell cultures.* A sample of at least 5 ml of each plasma pool shall be tested for adventitious agents by inoculation into simian cell cultures. Similar volumes of the plasma pool shall likewise be tested in human cell cultures. The cell cultures shall be observed for at least 14 days.

Suitable simian cell cultures are prepared from the kidneys of *Cercopithecus* monkeys or from VERO cells. For the human cell cultures, either WI-38 or MRC-5 may be used.

The plasma pool passes the test if none of the cell cultures shows evidence of the presence of any adventitious agent attributable to the plasma pool.

3.5 *Concentration and purification*

The method used for the concentration of the HBsAg shall be approved by the national control authority. Such methods shall have been shown to concentrate the HBsAg.

Precipitation by ammonium sulfate and polyethylene glycol have been found suitable.

The methods used for purification of the HBsAg shall be approved by the national control authority. The purification procedure shall be such that the bulk of extraneous host substances shall be removed.

Purification has been effected by such methods as fractional precipitation, chromatographic techniques, and sequential isopyknic and rate zonal centrifugation (some of these only in combination with others). Additional peptic digestion has also been used for the removal of extraneous proteins.

After concentration and purification the protein content shall be measured. Assays for control of HBsAg shall be performed and the amount of antigen shall account for most of the protein. The lower limit of the HBsAg content per unit weight of total protein shall be determined by the national control authority.
It has been found suitable to measure the concentration of protein by means of the extinction coefficient $E_{280}^{1\%}$, by micro-Kjeldahl, or by the Lowry technique (5).

The concentration of HBsAg may be measured by quantitative tests (7) in comparison with a reference preparation.

3.6 Inactivation

The vaccine shall be inactivated by a method or methods approved by the national control authority.

Because inactivation with formaldehyde introduces an extra safety factor without markedly affecting immunogenicity, such an inactivation step is usually included.

In one country a concentration of 1:4000 formalin for 72 hours at 36 °C is used.

The method of inactivation shall be shown to be satisfactory by the inoculation of susceptible chimpanzees (see Appendix, page 155).

3.7 Tests on purified, inactivated HBsAg batches

3.7.1 Sterility tests

A volume of at least 10 ml of each batch shall be tested for sterility according to the requirements given in Part A, section 5 of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (4, p. 49).

3.7.2 Tests for HBsAg

The titre of HBsAg shall be determined by a serological test. The test used and the lower limit of concentration permitted shall be determined by the national control authority.

Quantitative tests should be carried out in parallel with tests on a reference preparation.

The concentration of HBsAg shall be related to the total protein.

The total protein may be measured by the extinction coefficient $E_{280}^{1\%}$, by micro-Kjeldahl, or by the Lowry test (5).
3.7.3 Tests for extraneous substances

Tests shall be made for the presence of blood group substances and other blood proteins by methods approved by the national control authority.

Agglutinins and agglutinogens are tested for by haemagglutination. Immuno-electrophoresis, agar gel diffusion, more sensitive immunoassays, and polyacrylamide gel electrophoresis have been used to test for other extraneous proteins.

The preparation shall be free from blood group substances.

The permitted concentration of serum proteins present in the vaccine shall be determined by the national control authority.

A test shall be made for the presence of DNA polymerase by a method approved by the national control authority.

This test is for the detection of DNA polymerase associated with hepatitis B virus.

The preparation must be free from DNA polymerase.

3.7.4 Test for antigen protein

The total hepatitis antigen protein content of the material shall be measured by a method approved by the national control authority.

The extinction coefficient $E_{280}^1$ microkjeldahl, and the Lowry method (5) have been found to be suitable for the purpose of measuring total protein. and the radioimmune assay (RIA) method can be used to measure how much protein is hepatitis antigen protein.

3.7.5 Tests for agent used for purification

A test shall be made for the presence of any potentially hazardous agent that may have been used in the course of purification of the HBsAg.

The method used and the permitted concentration shall be approved by the national control authority.

3.7.6 Test for free formaldehyde

If the antigen has been treated with formaldehyde then the material shall be tested for the presence of free formaldehyde. The
method used and the permitted concentration shall be approved by the national control authority.

3.8 Final bulk

The final bulk consists of one or more purified, inactivated HBsAg batches. Only those batches that have satisfied the requirements of Part A, section 3.7 shall be included in the final bulk.

3.9 Addition of adjuvant

Where the final bulk contains an adjuvant, the adjuvant and the concentration used shall be approved by the national control authority. Where aluminium salts are used, the concentration of aluminium shall not exceed 1.25 mg per single human dose.

At this stage more preservative may need to be added.

3.10 Tests on final bulk

3.10.1 Sterility tests

A volume of at least 10 ml of the final bulk shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5.2 of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (4, p. 48).

3.10.2 Tests for preservative

The final bulk shall be tested for the presence of preservative. The method used and the permitted concentration shall be approved by the national control authority.

3.10.3 Test for HBsAg

The titre of HBsAg in the final bulk shall be determined by a suitable quantitative serological procedure. The lower limit of antigenic content shall be approved by the national control authority.
3.10.4 *Test for total protein*

The total protein content per human dose of the final bulk shall be measured by a method approved by the national control authority. The limits of total protein shall be approved by the national control authority.

3.10.5 *Pyrogenicity test*

Each final bulk shall be tested for pyrogenicity by the intravenous injection of rabbits. Three or more healthy rabbits that have not been injected previously shall be used. Each rabbit shall receive, by injection into the ear vein, one human dose.

The criteria for passing the test shall be those specified in the International Pharmacopoeia (6, Appendix 43, p. 747).

3.10.6 *Test in chimpanzees*

The national control authority shall determine how many of the initial lots of vaccine shall be tested for the presence of infectious hepatitis viruses in chimpanzees. The test shall be approved by the national control authority.

In some countries the national control authority may take the responsibility for omitting this test. A test shown to be suitable is described in the Appendix.

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) (2, p. 16) shall apply.

Care should be taken to ensure that the material of which the container is made does not adversely affect the HBsAg under the recommended conditions of storage.

5. **CONTROL TESTS ON FINAL PRODUCT**

Samples shall be taken from each final lot for the tests in the following sections.
5.1 Sterility tests

The 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 (General Requirements for Sterility of Biological Substances) (4, p. 48).

5.2 Innocuity tests

Each final lot shall be tested for innocuity by appropriate tests in mice and guinea-pigs using parenteral injections. The tests shall be those approved by the national control authority.

5.3 Test for preservative

Each final lot shall be tested for the presence of preservative. The test used and the permitted concentration shall be approved by the national control authority.

5.4 Test for total protein

A test for total protein shall be made by a method approved by the national control authority.

5.5 Assay of adjuvant

Each final lot shall be assayed for the content of adjuvant. The method used and permitted concentration shall be approved by the national control authority.

5.6 Potency and identity test

The vaccine shall be identified as HBsAg by appropriate methods. An appropriate quantitative potency assay shall be performed on each final lot irrespective of how many filling lots are made.

A suitable test in mice is as follows:

Groups of 10–20 white Swiss mice, 5 weeks of age, are vaccinated intraperitoneally with graded doses of hepatitis B vaccine. The mice are bled 28 days later and the sera are kept separate. Antibody determinations are performed by a sensitive quantitative test such as radioimmunoassay. The data are analysed
according to seroconversion as well as according to the geometric mean titre of anti-HBs for each antigen dose. These tests should be carried out in parallel with tests on a reference preparation.

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) (2, p. 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) (2, p. 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) (2, p. 17) shall apply, with the addition of the following directive:

The leaflet accompanying the package shall include the following information:
— the nature and amount of any preservative, adjuvant, or stabilizer present in the vaccine;
— the volume of one recommended human dose;
— the amount of protein contained in one recommended human dose; and
— the amount of HBsAg contained in one recommended human dose.

9. DISTRIBUTION AND SHIPPING

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) (2, p. 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) (2, p. 19) shall apply.

10.1 Storage conditions and stability

Before being distributed by the manufacturing establishment, or before being issued from a depot for the maintenance of reserves of vaccines, all vaccines in bulk form or in final containers shall be kept at 5±3°C or lower if practicable. After distribution or issue, the vaccine shall be stored at a temperature not exceeding 8°C. The vaccine shall have been shown to maintain the HBsAg content of the human dose for a period equal to that between the date of issue and the expiry date.

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 HBsAg concentration or the date of the last satisfactory potency test, the date of this test being that on which the test system was inoculated.

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) (2, p. 19) shall apply.
The national control authority shall:
— approve the medical evaluation of donors;
— approve the methods of sterility control;
— approve the tests for HBsAg subtypes;
— approve the tests for HBsAg concentration and define its minimum value;
— approve the methods for concentration, purification, and inactivation;
— approve the tests for extraneous substances and total protein;
— approve the tests for the agents used for concentration and purification, free formaldehyde, and preservative;
— approve the test for the presence of infectious hepatitis viruses;
— approve the innocuity test in the final product; and
— approve the adjuvant assay and define its permitted concentration in the final product.

The national control authority shall also provide national reference preparations for the expression of activity of HBsAg contained in a given quantity of protein.

2. RELEASE AND CERTIFICATION

A hepatitis B 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 whether or not the final 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 HBsAg 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 hepatitis B vaccine between countries.

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ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice
and for supplying additional data relevant to these Requirements:
Dr V. Davey, Technical Director, Commonwealth Serum Laboratories, Parkville, Vic-
toria, Australia
Dr A. Gray, Merck Sharp & Dohme, West Point, PA, USA
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REFERENCES


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Appendix

SAFETY TESTING OF HEPATITIS B VACCINE

In addition to the tests for innocuity applicable to all vaccines, a test to provide some assurance of the absence of viable hepatitis viruses shall be made in chimpanzees. Ideally, these chimpanzees would have been under observation for at least six months and would satisfy the following conditions. They shall:

1. Be free from hepatitis B virus infection past or present as shown by sensitive techniques (negative tests for HBsAg, anti-HBs, and anti-HBc).
2. Have normal levels of aminotransferases at the time of starting the study.
3. Have had at least one normal liver biopsy taken within six weeks of the start of the study.
4. Be housed in adequate isolation quarters and attended by persons free from hepatitis B infection.

It would be desirable also to use animals that have never received blood or blood products of human origin.

Each final bulk of hepatitis B vaccine or a combination of material from several final bulks shall be injected into chimpanzees.

The number of final bulks represented in the sample should be determined by the national control authority.

In no case should this be more than five final bulks. The aliquots taken from the lots should be the same for each lot.

In one country each of a total of four acceptable chimpanzees are injected as follows:
1. Two each receive one vaccine dose by the intravenous route.
2. Two each receive 10 vaccine doses by the intravenous route.

The observation period of six months shall include:
(a) Weekly determinations of aminotransferases. These shall remain normal for each individual chimpanzee; if present, they shall be convincingly demonstrated to be unrelated to hepatitis B.
(b) Weekly determinations using sensitive serological tests for the markers of HBV infection.

c) Weekly weight determinations and daily checks of general health.

d) Biopsies for light microscopic examination to search for evidence of hepatitis shall be taken monthly and at any time chimpanzees show any abnormality.

If after six months' observation the four chimpanzees have shown normal aminotransferase values throughout with no histological evidence of hepatitis or serological evidence of hepatitis B, the vaccine passes the safety test.

The national control authority shall determine how many batches of vaccine shall be tested for safety in chimpanzees. It shall determine also at what stage the number of chimpanzees may be decreased to two or the test omitted altogether.

Whether or not chimpanzees are available, the national control authority must take the responsibility for granting permission for clinical trials in man that may be required to establish the suitability of each vaccine lot.
INTRODUCTION

Since the Requirements for Poliomyelitis Vaccine (Oral) were revised in 1971 (WHO Technical Report Series, No. 486, 1972, Annex 1) there has been an accumulation of data, particularly concerning the performance and evaluation of the neurovirulence test, which demand changes in the relevant requirement. Further changes required concern the tests on karyology of the human diploid cells when used for virus growth and the adoption of a standard method for the titration of the virus. It is considered that such changes are of sufficient significance and urgency for an addendum to the present requirements to be formulated.

In 1978 the WHO Group of Consultants on Poliomyelitis Vaccine made from the Sabin Strains recommended that in order to avoid future discordant results in the neurovirulence tests of poliomyelitis vaccines there should be acceptance of a WHO method of assessment of neurovirulence based on a standard procedure for the inoculation of the virus and the adoption of a common method of assessment of
activity. This was discussed by a group of experts and the appropriate amendment to the requirements was made.

In view of the difficulty of obtaining rhesus monkeys for the neurovirulence test, work has continued on the relative susceptibility of rhesus and cynomolgus monkeys. Although by the intraspinal test the cynomolgus monkeys are more susceptible to the virus, the inclusion of a neurovirulence reference preparation in each test has shown that the two species of monkeys give similar results when a comparison is made between the reference preparation and the vaccine. It was agreed that either of these species of Macaca monkeys could be used. Cercopithecus monkeys may also be used if the national control authority has given its approval.

**The Neurovirulence Test for the Assessment of Oral Poliovirus Vaccines**

In the past it has been considered important to observe the neurotropic activity of vaccines by means of both the intrathalamic and the intraspinal route of inoculation. Further experience with type 3 vaccines has shown, however, that the intrathalamic test should no longer be included in the WHO Requirements for Poliomyelitis Vaccine (Oral) as a mandatory test.

Recent experiments in three laboratories and retrospective reviews of the experience gained during almost 20 years of vaccine testing have shown that, as currently practised, both the intrathalamic and the intraspinal test do not utilize monkeys to the best advantage. Neither test is well designed to obtain the data necessary to permit a precise and statistically valid comparison between the activity of a vaccine and the reference preparation. The intrathalamic test suffers from the relative insusceptibility of the monkeys to the virus when it is injected by this route, and the intraspinal test, in using graded doses, is designed to measure a 50% end-point of virus activity, and inevitably results in a large proportion of negative monkeys. It is now clear that an intraspinal test based on 20 monkeys, in which the distribution of virus-specific lesions within the central nervous system is observed, can provide the information necessary for a more precise comparison of vaccines with the reference preparation.

It was concluded that in the revised neurovirulence test, as in other comparative biological assays, it was desirable to test the reference preparation at the same time as the test on the vaccine and not—as was previously permitted—simply within a three-month period. The
working reference preparation should be distributed free of charge to both control authorities and manufacturers. It is also recommended that before licensing the first vaccine lots both the national control authorities and the manufacturer should perform the revised test on the working reference preparation on at least two occasions to establish baseline data for future comparison with the production lots.

AMENDMENTS

Amendment 1

Part A, section 1.3 International standards and international reference preparations

Replace the first paragraph by the following:

"The WHO Group of Consultants on Poliomyelitis Vaccine made from the Sabin Strains has adopted three monotypic virus suspensions and one trivalent virus mixture for types 1, 2, and 3 as the international reference preparations for the control of virus titre (see Part A, sections 3.5.4 and 5.3). These preparations are dispensed in ampoules and held in the frozen state in the gas phase of liquid nitrogen and are in the custody of the National Institute for Biological Standards and Control, London. They are distributed free of charge to national control authorities for the purposes of establishing international working reference preparations for the control of virus titre in the vaccine.

A reference preparation (NC-1) for neurovirulence of type 3 virus is held by the Bureau of Biologics of the Food and Drug Administration, Bethesda, MD, USA. This virus suspension is distributed free of charge for the control of neurovirulence of type 3 vaccines. The neurovirulence reference preparations for types 1 and 2 virus are under investigation. When approved by the collaborating laboratories they will be made available."

Amendment 2

Part A, section 3.2.2 Monkey kidney-cell cultures for vaccine production

Replace the paragraph in small type by the following:

"Suitable antibiotics in small concentrations may be used but penicillin and streptomycin may not be used. Nontoxic pH indicators may be added, such as phenol red in a concentration of 0.002%. Only those substances that have been approved by the national control authority may be added."
Amendment 3

Part A, section 3.5.4  *Virus concentration*

*Replace the whole section by the following:*

"The determination of the amount of infective virus per ml of filtered bulk suspension shall be made in cell cultures of the Hep-2 (Cincinnati) cell line. This determination should be made in terms of TCID₅₀ per ml in microtitre plates in parallel with the determination of the virus concentration of a reference preparation containing the same virus type of known virus titre (see Part A, section 1.3). The determination of the number of TCID₅₀ per ml shall be based on the use of twofold dilution steps with 12 microtitre wells per dilution (see Appendix 1).

Other methods which have been shown to be of equivalent sensitivity as described above may be used."

Amendment 4

Part A, section 3.5.5.1  *Tests in monkeys for neurovirulence*

*Replace the whole section by the following:*

"Monkeys used for neurovirulence tests shall be those that conform to the relevant requirements in Part A, section 3.1.2 and weigh between 1.5 kg and 3.0 kg. The pathogenicity of the filtered bulk suspension for *Macaca* or *Cercopithecus* monkeys shall be tested in comparison with that of the seed lot or a reference virus preparation for neurovirulence testing (see Part A, section 1.3) by inoculation into the lumbar region of the central nervous system. A pre-injection serum sample obtained from each monkey shall be shown to contain no neutralizing antibody in a dilution of 1:4 when tested against no more than 1000 TCID₅₀ of each of the three types of poliovirus.

(1)  *Number of monkeys*

The number of monkeys inoculated shall be such that at least 20 valid monkeys shall be included in the evaluation of the vaccine. Similarly, at least 10 valid monkeys shall be included for the reference preparation.

---

"¹ A reference to a suitable source of cells may be obtained from Chief, Biologicals, World Health Organization. Geneva, Switzerland."
To have 20 and 10 valid monkeys available for testing, at least 24 and 12 monkeys respectively should be inoculated.

A valid monkey is one in which there is microscopic evidence for the inoculation of the virus into the grey matter of the lumbar enlargement (see (3) below).

The monkeys are sedated; phencyclidine hydrochloride or ketamine hydrochloride has been shown to be suitable.

(2) **Virus content of seeds, vaccines, and reference preparations inoculated**

The virus content of the reference preparation shall be between 7.3 and 8.0 \( \log_{10} \) TCID\(_{50}\) per ml. The virus titre of the seed or vaccine shall be adjusted when necessary to ensure a virus content within this range, and this will be inoculated as the higher dose of vaccine. A tenfold dilution of this vaccine dose shall be made and inoculated as the lower dose of vaccine. The reference vaccine shall be inoculated both undiluted and diluted to \(10^{-1}\).

The monkeys inoculated shall be divided into two equal groups, one receiving the lower dose and the other the higher dose.

The monkeys receiving vaccine and reference preparation should be matched as far as possible for sex, weight, and origin. The accumulation of data with the reference preparation may be achieved by the inoculation of 80 monkeys.

The virus titre of a sample of the seed or vaccine to be inoculated into the monkeys shall be determined. A standard method as determined by an international collaborative study of the titration of the virus preparations shall be used. (See Part A, section 3.5.4.)

A post-inoculation virus titre of the vaccine inoculated should be determined in order to check the virus content of the inoculum.

(3) **Method of inoculation**

The monkeys shall be inoculated by a method that places the whole inoculum within the grey matter of the lumbar enlargement. The needle shall be inserted between the first and second lumbar vertebrae and passed ventrally until the needle is in the cord.

It is important to observe the muscle twitch of one foot and leg or both feet and legs of the monkey when the needle enters the cord. Without further movement of the needle the second motor response (fibrillation of the muscles) should be observed during the inoculation of the virus. If this is not observed the needle should be withdrawn and a further inoculation made.
A needle measuring 0.5–0.6 mm by 25 mm shall be used and the inoculum of 0.1 ml shall be given over a period of not less than 5 seconds.

The object is to place the whole inoculum in the cord with as little mechanical trauma as possible.

(4) Observation of monkeys

All monkeys shall be observed for 17–22 days for symptoms suggestive of poliomyelitis or other virus infection. Monkeys that survive the first 24 hours but die before the eleventh day after inoculation shall be autopsied to determine whether poliomyelitis has been the cause of death. Those that have died due to causes other than poliomyelitis shall be excluded from the evaluation.

Animals which become moribund or which are severely paralysed should be killed and autopsied.

All monkeys that survive the observation period shall be autopsied.

For the test to be valid, at least 80% of the animals in each group shall have remained healthy and survived the observation period.

(5) Number of sections examined

Histological examination shall be made of at least the lumbar cord, the cervical cord, the lower and upper medulla oblongata, and the mesencephalon, as well as the motor cortex of each monkey.

Sections shall be cut at a thickness of 15 μm and stained by galloccyanin. The minimum number of sections examined shall be as follows:
— 12 sections taken throughout the lumbar enlargement;
— 10 sections taken throughout the cervical enlargement;
— 2 sections taken from the medulla oblongata;
— 1 section from the pons and cerebellum;
— 1 section from the mid-brain; and
— 1 section each from the left and the right of the thalamus and cerebral cortex.

In some countries Nissl staining is permitted.

(6) Scoring of virus activity

In the evaluation of virus activity in the hemisections of the spinal cord and brain stem a system of scoring the severity shall be used.
The specific damage, whether cellular infiltration or destruction of neurons, is important. The scoring system shall be:

1. Cellular infiltration only.
2. Cellular infiltration with minimal neuronal damage.
3. Cellular infiltration with extensive neuronal damage.
4. Massive neuronal damage with or without cellular infiltration.

The scores obtained shall be recorded on a standard form (see Appendix 2).

(?) Evaluation of neurovirulence test

The evaluation of the virus activity in the vaccine and reference preparation shall be compared by the activity in the lumbar enlargement of the cord and the degree of spread of activity from this region to the cervical enlargement and the brain.

The acceptance or rejection of the vaccine shall be based on the total scoring of all test animals. Individual animals showing unusually high activity either in the lumbar region or by spread shall also be taken into consideration in the final evaluation.

The filtered bulk passes the test if the animals show evidence of valid inoculation trauma and if none of the clinical and histopathological findings shows a statistically significant difference in pathogenicity between the vaccine virus and the reference material.”

Amendment 5

Part B, section 1 General

Replace the third paragraph by the following:

“The detailed test procedures for production and control shall be those approved by the national control authority. The national control authority shall obtain the international reference preparations for virus titre and establish national working reference preparations by comparison with them.

The national control authority shall obtain the international working reference preparations for neurovirulence. The control authority and the manufacturers should each carry out at least two tests to obtain the necessary baseline data for comparison with the neurovirulence of test vaccines.
The national control authority should encourage the use of the standard form for the reporting of data of virus activity in the sections taken for histopathological examination.

Since all manufacturers and control authorities will, for the first time, be using the same neurovirulence reference preparations it would be helpful to all laboratories to have accumulated data on the results obtained with the reference preparation. The within-laboratory and between-laboratory data would be informative and in order to build up such a data bank the control authorities are requested to report their data to WHO (Chief, Biologicals) for the analysis of these data."

Amendment 6

Part C, section 1.4 Terminology

Add the following:

"Manufacturer's working cell bank (MWCB): a quantity of cells derived from a single ampoule of the cell seed and of uniform composition stored frozen at ~70°C or below in aliquots, one of which would be used for the production of each single harvest.

In normal practice such a seed culture (or ampoule) is issued to manufacturers at or near the eighth population doubling level (PDL). This is expanded by serial subculture up to a PDL selected by the manufacturer at which point the cells are combined into one or more pools and preserved cryogenically to form the MWCB. One or more of such ampoules from a pool would be used for the production of a single harvest.

Production cell culture: a collection of cell cultures at the population doubling level used for virus growth that have been derived from a single ampoule of the MWCB."

Amendment 7

Part C

Add the following as sections 3.1.3, 3.1.4, and 3.1.5:

"3.1.3 Tumorigenicity

The cells at the production level shall also be shown to be free from potential tumorigenicity by appropriate tests in animals approved by the national control authority.
Suitable tests using immunosuppressed animals may be made as follows. Approximately $10^6$ viable cells obtained from cultures at the same passage level as those used for vaccine production are injected into: (a) newborn mice or hamsters treated with antilymphocytic serum; or (b) athymic mice (nude nu/nu genotypes); or (c) thymectomized mice irradiated and bone marrow reconstituted (T-B+). Some of the same group of animals should be inoculated with a similar dose of HeLa or KB cells and it should be shown that tumour formation is caused by the inoculation of the neoplastic tissue, thus demonstrating the ability of the strain of animals to give rise to tumours. The animals should be observed for not less than three weeks. Any other test using animals treated with immunosuppressive agents and with equal sensitivity to neoplastic cells may be used.

Only those cell seeds shown not to be tumorigenic shall be used.

3.1.4 Chromosomal characterization and monitoring

3.1.4.1 Chromosomal characterization. At least four samples shall be examined as described in Part C, section 3.1.4.2, at approximately equal intervals over the life-span of the cell line during serial cultivation. Each sample shall consist of 1000 metaphase cells.

It is also recommended that photographic reconstruction should be employed in the preparation of chromosome-banded karyotypes of 50 metaphase cells per 1000-cell sample using either G-banding or Q-banding techniques. This constitutes 5% of the sample and no specific limits for acceptability are yet recommended. The incidence of karyotypic abnormalities (pseudo-diploidy, inversions, translocations, etc.) that are detectable with the greater resolution provided by banding should be evaluated when a larger data base than is at present available has been accumulated.

3.1.4.2 Chromosomal monitoring—preparation and testing. For the determination of the general character of each pool in the MWCB, a minimum of 500 cells in metaphase shall be examined at the production level or at any passage thereafter for frequency of polyploidy and for exact counts of chromosomes, frequency of breaks, structural abnormalities, and other abnormalities such as despiralization or marked attenuations of the primary or secondary constrictions.

For vaccine production, examination of the cells is usually made between the twenty-seventh and thirty-third population doubling. The national control authority should determine the level of cell population doubling allowable.
For cells examined in metaphase the upper limits\(^1\) of acceptability (upper fiducial limits at 95% (Poisson)) for abnormalities are for a 1000- and 500-cell sample examined.

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>1000 cells</th>
<th>500 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatid and chromosome breaks</td>
<td>47/1000</td>
<td>26/500</td>
</tr>
<tr>
<td>Structural abnormalities</td>
<td>17/1000</td>
<td>10/500</td>
</tr>
<tr>
<td>Hyperploidy</td>
<td>8/1000</td>
<td>5/500</td>
</tr>
<tr>
<td>Hypoploidy</td>
<td>180/1000</td>
<td>90/500</td>
</tr>
<tr>
<td>Polyploidy</td>
<td>30/1000</td>
<td>17/500</td>
</tr>
</tbody>
</table>

All cells showing abnormalities shall be subjected to detailed examination, and records shall be maintained of the detailed criteria applied to particular abnormalities evaluated in the karyotype analysis.

Stained slide preparations of the chromosomal monitoring of the working cell bank pool, or photographs of these, shall be maintained permanently as part of the record of the batch of vaccine and for monitoring successive batches made from that cell pool.

It is desirable that a portion of the sample of pooled cell substrate removed from the culture vessels should be stored frozen so as to retain viability. This would be available for future reference for karyology or for any other purpose relating to the batch of vaccine.

Only those cell pools of the MWCB that have normal karyology shall be used for vaccine production.

3.1.5 Identity test of the cells

In some countries tests for characterizing HLA surface antigens are carried out in addition to chromosome monitoring."

*Renumber Part C, section 3.1.3 as 3.1.6, and section 3.1.4 as 3.1.7.*

**Amendment 8**

Part C

*Replace sections 3.2, 3.2.2, 3.2.3, and 3.2.3.1 by the following:*

"\(^1\) These upper limits are based on extensive experience with the examination of WI-38 and MRC5 cells reported to and examined by the Ad Hoc Committee on Karyological Controls of Human Cell Substrates, which met in 1978 at Lake Placid, NY, USA. These values will not necessarily be applicable if another human cell strain is used."
3.2 Production of cell culture

At least 5% of the cell suspension (not less than 500 ml) of the concentration employed for seeding the vaccine production cultures shall be used to prepare control cultures.

In some countries in which the technology of large-scale production has been developed the national control authority should determine the size of the sample of cells to be examined.

The treatment of cells set aside as control material shall be similar to that of the production cell cultures, but they shall remain uninoculated as control cultures for the detection of extraneous viruses.

These control cell cultures shall be incubated under the same conditions as the inoculated cultures for at least two weeks or until the time of the last harvest of the production cultures—whichever is the later—and shall be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures shall have had to be discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures shall be examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section shows evidence of the presence in a control culture of any adventitious agent, the virus grown in the corresponding inoculated cultures shall not be used for vaccine production.

3.2.1 Test for haemadsorbing viruses

At the end of the observation period, cells comprising 25% of the control cells shall be tested for the presence of haemadsorbing viruses, using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage shall not have exceeded 7 days, and the temperature of storage shall have been in the range of 2–8 °C.

In some countries the national control authority requires that tests for haemadsorbing viruses should be made in addition, using other types of red cells including those from humans (blood group IV, O), monkeys, and chickens (or other avian species). The cultures should be examined at 3–5 days and again at 12 days. All tests should be read after incubation for 30 min at 0–4 °C and again after a further incubation for 30 min at 20–25 °C. The test with monkey red cells should be read once more after yet another incubation for 30 min at 34–37 °C.
3.2.2 Identity test

At the production level the cells shall be identified as human by
tests approved by the national control authority.

Suitable tests are isoenzymes, HLA, or other immunological
tests or karyotype of at least one metaphase spread of chromo-
somes."

Amendment 9

Part C

Delete sections 3.2.4, 3.2.4.1, and 3.2.4.2

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ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice
and for supplying additional data relevant to the formulation of these modifications:

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THE ASSAY METHOD FOR THE DETERMINATION OF THE VIRUS CONTENT OF FINAL BULK SUSPENSIONS OF ORAL POLIOVIRUS VACCINE

The preparation to be assayed and the reference preparation are diluted in Earle's balanced Basal Medium Eagle (BME) containing 0.13% sodium bicarbonate and 4% of fetal calf serum with penicillin (200 units/ml) and streptomycin (100μg/ml).

It is convenient to make tenfold dilution steps of the virus suspensions initially to about 10^{-6} or 10^{-7}, but for those dilutions which are to be inoculated into cell cultures the dilutions should be prepared in twofold steps. A preliminary assay may be required to ensure that in the test the dilution range selected encompasses at least 3 dilutions, infecting between 10% and 90% of the cultures inoculated.

Groups of 12 flat-bottomed wells in a microtitre plate are inoculated with 0.1 ml of each of the selected dilutions of virus followed by 0.1 ml of a cell suspension containing approximately 5×10^9 cells of the Hep-2 (Cincinnati) line. (See below for the technique of sub-cultivation of the cells.) The plates are sealed and incubated at 35–36°C for 7 days.

The cultures are examined for the presence of a specific viral cytopathic effect on days 3–5 and again on day 7. The observations are recorded and the TCID_{50} is calculated on the basis of the observation on day 7. The assay should be repeated on at least two occasions and a mean taken. For the estimated potency of the monovalent lot to be accepted as valid the observed mean for the reference should be within 0.5 log_{10} TCID_{50} of the established mean for this preparation.

Cultivation of Hep-2 Cincinnati Cells

1. Receipt of a cell seed

A 150-ml bottle contains a confluent, or nearly confluent, sheet of cells of approximately 50 cm^2. It is filled with growth medium (i.e.,
BME to which serum and antibiotics have already been added) to prevent the loss of cells in transit. On arrival, decant all except about 15 ml of the medium and incubate the culture at 37°C till a confluent sheet has been formed. Stored at 40°C, the decanted medium may be used for further subcultivation of cells.

2. Subcultivation of cells

2.1 When the cell sheet is quite confluent pour off medium and rinse twice with 5 ml of trypsin solution (see below for reagents). Pour off the trypsin and allow the bottle to remain at room temperature (or incubate at 37°C) until the cells begin to detach from the glass.

2.2 Add about 5 ml of pre-warmed BME (see below for reagents). Agitate the bottles so that the medium washes back and forth and causes complete release of cells from the bottle wall. When release is complete, aspirate thoroughly with a 5-ml pipette with a bulb in order to effect maximum disaggregation of cells.

2.3 Add a further 55 ml of medium and either use the suspension for a virus assay or distribute one-quarter of the total volume of cell suspension to each of 3 additional bottles having the same surface area as the “parent” bottle and incubate the cultures at 37°C. This is a 1:4 “split” with cells at a concentration of approximately 5 x 10⁶ cells per ml.

2.4 Subculture cells (1:4 “split”) at intervals of 3–4 days, when the cells growing normally will have formed confluent sheets.

2.5 It is important to keep a record of the number of passages through which the cells have been propagated. The virus sensitivity of the cells may decrease during subcultivation.

Reagents

1. Trypsin: Final concentration 0.25% in Balanced Salt Solution (BSS) (Hanks’, Earle’s, or Phosphate Buffered Saline (PBS)), pH about 7.8.
2. **Medium:** Basal Medium Eagle (BME)

   - in Earle’s BSS ............ 95% (by volume)
   - Fetal calf serum ............ 5% (by volume)

**Antibiotic(s):**

- Penicillin (100 units/ml)
- Streptomycin (100 μg/ml)

To buffer, add sodium bicarbonate to a final concentration of 0.088%. Pre-warm medium and trypsin solution to 37°C before use.

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**Appendix 2**

**FORM ON WHICH TO REPORT THE SCORE OF VIRUS ACTIVITY FOR EACH SECTION FROM EACH MONKEY IN THE NEUROVIRULENCE TEST**

Although the test requires that at least 10 valid monkeys should be inoculated with the higher and lower doses of virus, provision is made in the form for recording results for 12 monkeys that may be inoculated and survive the test. A separate form will be required for each dilution of vaccine and reference preparation.

In the forms the scoring system used for all sections from all areas is:

1. Cellular infiltration only.
2. Cellular infiltration with minimal neuronal damage.
3. Cellular infiltration with extensive neuronal damage.
4. Massive neuronal damage or without cellular infiltration.

**Final bulk No. ____________________________**

**Date of certification ________________________**

I certify that the above final bulk complies with the WHO requirements for tests in monkeys for neurovirulence (WHO Technical Report Series, No. 658, 1981; Amendment 4 to Part A, section 3.5.5.1, Annex 1, of WHO Technical Report Series, No. 486, 1972).

**Signature ________________________________**

**Name (typed) ______________________________**
**Appendix 2 (continued)**

INTRASPINAL NEUROVIRULENCE TEST IN MONKEYS: HISTOLOGY

All data from all monkeys must be recorded, which may require expansion of this form. Clinical signs of paralysis must be recorded on a separate form.

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<thead>
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<th>Test No.</th>
<th>Type</th>
<th>Final Bulk No.</th>
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</table>

Post inoculation Titre: $\log_{10} \text{TCID}_{50}/\text{ml}$  

Dilution  

Date of inoculation  

Date of end of test

<table>
<thead>
<tr>
<th>M</th>
<th>V</th>
<th>Histological lesions of poliomyelitis</th>
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<td></td>
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<td>Lumbar sections</td>
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</tbody>
</table>

**KEY:**
- M = Monkey number
- V = Validity of inoculum
- L = Left
- R = Right
- Av = Average
- Med = Medulla
- Cb = Cerebellum
- P = Pons
- M = Midbrain
- T = Thalamus
- Co = Cortex

---

**Signature**

**Date**

**Name (typed)**
INTRODUCTION

The WHO Expert Committee on Biological Standardization, in 1975, adopted the Requirements for Meningococcal Polysaccharide Vaccine, which were published in its twenty-seventh report (1, p. 50). At that time it was recognized that the technology involved in the quality control of such vaccines was changing rapidly but the Requirements were written because meningococcal meningitis is a serious problem in many countries and national health authorities, particularly in the developing countries, had requested guidelines for quality control.

During the year following the formulation of the Requirements, progress had been made in the preparation of polysaccharides of higher molecular weight (greater potency) for both Group A and Group C. Furthermore, a greater purification was achieved by the removal of endotoxin so that it became possible to inoculate 10 times the quantity of the polysaccharides into rabbits without a significant rise in temperature. Accordingly, an addendum to the Requirements to take these developments into consideration was published in the twenty-eighth report of the Expert Committee on Biological Standardization (2, p. 52).

Further amendments were made and published in the twenty-ninth report of the Expert Committee on Biological Standardization (3, p. 94) in order to take into consideration the need to add lactose as a stabilizer and thereby prevent thermal depolymerization. It was
also reported that much research was in progress on the production of polysaccharides from Groups Y, 29E, and W135, but infections by these Groups occurred at a lower frequency than those by Group A and Group C.

Such research has now shown that the polysaccharides from each of these three additional Groups can be isolated and stabilized and they have been characterized. The incidence of infections by Groups Y and W135 strains are increasing in some countries. They now account for more than 10% of the cases and are particularly virulent. It is important, therefore, to make provision for their inclusion in the vaccine, and in so doing a number of the requirements will need amendment. It is recommended that where the infectious agent causing meningitis has not been grouped a quadraspecific vaccine should be used.

In order to avoid reference to several publications, the amendments published in the twenty-eighth and twenty-ninth reports of the Expert Committee on Biological Standardization are included in the present amendments.

AMENDMENTS

Amendment 1

Part A, section 1.1  International name and proper name

Replace the whole section by the following:

"The international name shall be 'Vaccinium meningitidis cerebrospinalis' followed in parentheses by the serogroup specificity, thus: polysaccharide Group A, Group C, Group Y, and/or Group W135. 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."

Amendment 2

Part A, section 1.2  Descriptive definition

Replace the first sentence by the following:

"Vaccinium meningitidis cerebrospinalis shall consist of purified Group A, Group C, Group Y, and/or Group W135 meningococcal polysaccharides."
Amendment 3

Part A, section 3.1.1 Strains of Neisseria meningitidis

Replace the whole section by the following:

“The strains of Neisseria meningitidis Group A, Group C, Group Y, and Group W135 used for preparing polysaccharide shall be approved by the national control authority. They shall also have been shown to be capable of producing polysaccharide known to be safe and effective in man.

The following strains have been shown to be suitable: for Group A polysaccharide—A1, M1027; for Group C polysaccharide C11, C2241; for group Y polysaccharide—Y (Slaterus), 6306Y, IM2261; for Group W135—W135 6308, S. 4383, IM2263.”

Amendment 4

Part A, section 3.4

Replace the section heading by the following:

“Chemical and immunochemical requirements for lots of purified polysaccharide”

Amendment 5

Part A, section 3.4.1 Protein content

Replace the whole section by the following:

“Each lot of purified polysaccharide shall contain less than 10 mg of protein per gram of polysaccharide for Groups A and C organisms and less than 50 mg of protein per gram of polysaccharide for Groups Y and W135 as determined by the method of Lowry et al. [reference: LOWRY, O.H. ET AL. Journal of biological chemistry, 193: 265 (1951)] using bovine plasma albumin as a reference or other methods approved by the national control authority.”

Amendment 6

Part A, section 3.4.2 Nucleic acid content

Replace the whole section by the following:

“Each lot of purified polysaccharide shall contain less than 10 mg of nucleic acid per gram of polysaccharide for Groups A and C organ
isms and less than 20 mg of nucleic acid per gram of polysaccharide for Groups Y and W135 as determined by spectrophotometry, assuming that the absorbance of a 10 g/l solution of nucleic acid contained in a cell 1 cm wide at 260 nm is 200.”

**Amendment 7**

Part A, section 3.4.3 O-acetyl content

*Replace the whole section by the following:*

“The O-acetyl content of the polysaccharides from the different strains shall be equal to or greater than 2.0 mmol/g of polysaccharide for Group A, 1.5 mmol/g of polysaccharide for Group C, and 0.3 mmol/g of polysaccharide for each of the Groups Y and W135. The O-acetyl content shall be determined by the method of Hestrin [reference: HESTRIN, S. *Journal of biological chemistry*, **180**: 249 (1949)], using acetylcholine chloride as the reference preparation.”

**Amendment 8**

Part A, section 3.4.5 Sialic acid content

*Replace the whole section by the following:*

“The sialic acid content of the purified polysaccharide, calculated as free N-acetylneuraminic acid (molecular weight 309), shall be not less than 800 mg/g of the dry weight of the isolated product for Group C and not less than 560 mg/g for each of the Groups Y and W135. The determination shall be done by the method of Svennerholm [reference: SVENNERHOLM, L. *Biochimica et biophysica acta*, **24**: 604 (1957)] using sialic acid, N-acetylneuraminic acid, as the reference preparation.”

**Amendment 9**

Part A, section 3.4.6 Molecular size

*Replace the paragraph in large type by the following:*

“The molecular size of each lot of purified polysaccharide shall be estimated by gel filtration using Sepharose 4B or Sepharose CL-4B. Chromatography shall be carried out in a solvent having an ionic strength of 0.2 mol/kg. The molecular size shall be determined by
measuring the recovery of the polysaccharide eluted before a K_D of 0.50 is reached. At least 65% of the Group A, 75% of the Group C, 80% of the Group Y, and 80% of the Group W135 polysaccharides shall be recovered from the column before a K_D value of 0.50 is reached.”

Amendment 10

Part A

Add the following new section:

“3.4.7 Test for serological identity and specificity

Each lot of purified monospecific polysaccharide shall be tested for serological identity and specificity by a test approved by the national control authority.

Tests such as haemagglutination inhibition, immunoprecipitation, counter-immunoelectrophoresis, radioimmunoassay, ELISA, or rocket electrophoresis have been shown to be suitable.

The monospecific polysaccharide of Group A or C or Y or W135 organisms shall be shown to contain less than 1% by weight of any heterologous N. meningitidis polysaccharides during production by the manufacturer.

An immunoprecipitation test has been shown to give reliable results. In some countries the haemagglutinin inhibition test has been used but it has been shown to give some unreliable results. An immunochemical test is therefore to be preferred. Tests such as radioimmunoassay, ELISA, rocket or counter-current immuno-electrophoresis have been shown to be suitable.”

Amendment 11

Part A, section 3.5 Preparation of final bulk

Amendment 2 of the 1977 Addendum remains unaltered, as follows:

“The final bulk shall be prepared either from a single lot of purified polysaccharide or from several pooled lots. The polysaccharide shall be dissolved under aseptic conditions in a sterile solution suitable for freeze-drying and free of pyrogenic substances. A stabilizer shall be added, the substance used and its concentration being subject to approval by the national control authority.
The mixture shall be sterilized by membrane filtration.
Membranes with a pore size of 0.22 μm have been found satisfactory.
A suitable stabilizer is lactose at a concentration of 2.5–5.0 mg per human dose of polysaccharide. It is important to calculate the concentration of lactose on the basis of the anhydrous lactose molecule and not on the basis of the pentahydrate, which is the form most commonly available.”

Amendment 12

Part A, section 3.5.1  Sterility test on the final bulk
Delete the whole section.

Amendment 13

Part A, section 3.5.2  Test for serological specificity
Re-number as section 3.5.1 and replace by the following:

“3.5.1  Test for serological identity
The final bulk of the monospecific or polyspecific meningococcal polysaccharides shall be tested for serological identity and for the absence of heterologous polysaccharides by the test described in Part A, section 3.4.7.”

Amendment 14

Part A, section 4, Filling and drying
Replace the last sentence by the following:
“The stabilized dried vaccine shall be stored at a temperature of 2–8°C.”

Amendment 15

Part A, section 5.3  Concentration of polysaccharide
Replace the whole section by the following:
“At least one final container shall be checked to determine that it contains the stated amount of polysaccharide. Monospecific vaccines
shall contain at least 75 mg of phosphorus per gram of Group A polysaccharide, 750 mg of sialic acid per gram of Group C polysaccharide, or 520 mg of sialic acid per gram of the polysaccharides from Group W135 or Group Y. In multispecific vaccines in which the polysaccharides cannot be distinguished chemically this shall be done by a quantitative immunochemical test approved by the national control authority. The final container shall contain the declared content of each Group-specific polysaccharide ± 30%, using the purified polysaccharides incorporated in the vaccine as a reference.”

Amendment 16

Part A, section 5.5.1  Pyrogenicity test

Replace

“Group A vaccine, 0.025 µg
Group C vaccine, 0.025 µg
combined Groups A and C vaccine, 0.050 µg”

by the following:

“Group A vaccine, 0.025 µg
Group C vaccine, 0.025 µg
Group Y vaccine, 0.025 µg
Group W135 vaccine, 0.025 µg
A bispecific vaccine, 0.05 µg
A trispecific vaccine, 0.075 µg
A quadrasipecific vaccine, 0.10 µg”

Amendment 17

Part A, section 5.6  Estimation of molecular size

Replace the whole section by the following:

“The molecular size of the polysaccharide in at least one final container from each filling lot shall be determined by Sepharose 4B or Sepharose CL-4B gel filtration as outlined in Part A, section 3.4.6. For both monospecific and multispecific vaccines in which the polysaccharides cannot be distinguished chemically, the criteria of Part A, section 3.4.6 shall apply.”
When the column eluates are evaluated by an immunochemical method (see Part A, section 5.3) the major peak of each group polysaccharide shall elute with a $K_D$ value of 0.40 or less."

_Delete footnote 1._

**Amendment 18**

Part A, sections 5.7 and 5.8

*Replace these sections by the following:*

"**5.7 Test for residual moisture**

The moisture content of the dried material shall be determined as indicated in Part A, section 3.4. The method used for the determination of the moisture content shall be approved by the national control authority.

The test shall be performed on 1 vial per 1000 up to a maximum of 10 vials but not less than 5 vials taken at random throughout the filling lot. The average residual moisture shall be not greater than 2.5% and no vial shall have a residual moisture content of 3% or greater.

**5.8 Storage**

The stabilized freeze-dried vaccines shall be stored at a temperature of 2–8°C."

**Amendment 19**

*Amendment 8 of the 1977 Addendum remains unaltered as follows:*

"Section 8, on labelling, should reflect the fact that for stabilized vaccines it is no longer necessary to store Group A polysaccharide at −20°C or lower. Delete the last paragraph and replace by the following:

Furthermore, the label on the container, or the label on the carton enclosing several containers, or the leaflet accompanying the container shall contain the following additional information:

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— a statement that the stabilized vaccines shall be stored at 2–8 °C;  
— a statement that the reconstituted stabilized vaccine shall be stored at 2–8 °C and shall be discarded if not used during the day on which it is reconstituted.”

Amendment 20

Replace Amendment 9 of the 1977 Addendum by the following:

“10.1 Storage conditions

The manufacturer shall recommend such conditions of storage and shipping as will ensure that the vaccine conforms to these requirements until the expiry date as stated on the label. The Group A, C, Y, and/or W135 vaccine shall be stored at a temperature between 2 °C and 8 °C.”

Amendment 21

Replace Amendment 10 of the 1977 Addendum by the following:

“10.2 Expiry date

The expiry date for dried bulk polysaccharide when stored at –20 °C or below shall not be more than 5 years from the date of harvest. The expiry date of the stabilized vaccine in the final containers when stored at 2–8 °C shall be not more than 2 years from the date of issue. The manufacturers shall provide data to confirm the stability of their vaccine.

A further test for molecular size may be made and if it is found to be satisfactory the national control authority may wish to allow a further storage period of one year.”

Amendment 22

Amendment 11 of the 1977 Addendum remains unaltered as follows:

“in the Appendix to the Requirements, section 3, subsection (4), delete the last sentence of paragraph (d), together with footnote 1.”

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Amendment 23

Part B, section 3. Reactivity and immunogenicity of vaccine in man

Replace the third paragraph of the requirements printed in small type by the following:

"Samples of sera from each subject taken immediately prior to injection and again 2–4 weeks after immunization should be assayed for bactericidal antibodies. The bactericidal assay should be performed with paired sera from each subject in serial two-fold dilutions against a suitable strain for each of the polysaccharides contained in the vaccine. The antibody titre should be expressed as the reciprocal of the highest dilution that effects 50% or greater killing of the test organisms. The antibody titres of the sera from at least 90% of the subjects should show a four-fold or greater rise after immunization. If the sera from less than 90% but more than 80% of the subjects show such a rise, one retest of the product may be allowed, but in such a case the sera from at least 90% of all subjects in the two tests combined should show a fourfold or greater antibody increase."

ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice and for supplying additional data relevant to the formulation of these modifications:

Dr F. André, Smith Kline RIT, Rixensart, Belgium
Dr F. Arminjon, Mérieux Institute, Charbonnières-les-Bains, France
Dr R. Austrian, University of Pennsylvania, Philadelphia, PA, USA
Dr G. Ayme, Mérieux Institute, Charbonnières-les-Bains, France
Dr E. C. Beuvery, National Institute for Public Health, Bilthoven, Netherlands
Dr G. Y. Causse, Chief, Bacterial and Venereal Infections, WHO, Geneva, Switzerland
Dr P. Crooy, Smith Kline RIT, Rixensart, Belgium
Professor S. G. Dzagurov, Tarasevich State Institute for the Standardization and Control of Medical Biological Preparations, Moscow, USSR
Dr C. E. Frasch, Bureau of Biologics, Food and Drug Administration, United States Public Health Service, Bethesda, MD, USA
Dr C. B. Gerichter, Ministry of Health, Jerusalem, Israel
Dr E. Gotschlich, Rockefeller University, New York, USA
Dr C. Guthrie, Production Director, Commonwealth Serum Laboratories, Parkville, Victoria, Australia
Dr J. Henrichsen, State Serum Institute, Copenhagen, Denmark
Dr M. Hilleman, Merck Institute for Therapeutic Research, West Point, PA, USA
Dr M. Huet, National Health Laboratory, Montpellier, France
Professor D. Ikić, Institute of Immunology, Zagreb, Yugoslavia

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Professor L. Jännes, Chief, Production Laboratory, Central Public Health Laboratory, Helsinki, Finland
Dr J. Jeljaszewicz, National Institute of Hygiene, Warsaw, Poland
Professor G. Laurell, University of Uppsala, Sweden
Dr P. Lemoine, Chief, Vaccine Control Section, Institute of Hygiene and Epidemiology, Brussels, Belgium
Dr R. de Neys, Smith Kline RIT, Rixensart, Belgium
Professor F. Oberdoerster, State Institute of Serum and Vaccine Testing, Berlin, German Democratic Republic
Professor F. Pocchiari, Director-General, Higher Institute of Health, Rome, Italy
Mr K. Pope, Merck Sharp & Dohme, Zürich, Switzerland
Dr G. del Real, Llorente Institute, Madrid, Spain
Dr J. B. Robbins, Bureau of Biologics, Food and Drug Administration, United States Public Health Service, Bethesda, MD, USA
Dr E. J. Ruitenberk, National Institute for Public Health, Bilthoven, Netherlands
Professor M. Saletti, SCLAVO Institute for Sera and Vaccines, Siena, Italy
Dr R. SchneeBon, Bureau of Biologics, Food and Drug Administration, United States Public Health Service, Bethesda, MD, USA
Mr J. R. Thayer, National Biological Standards Laboratory, Canberra, Australia
Dr R. H. Tijssen, National Institute for Public Health, Bilthoven, Netherlands
Dr A. F. Woodhour, Merck Sharp & Dohme, West Point, PA, USA

REFERENCES

INTRODUCTION

Oral anticoagulant drugs derived from coumarin (and sometimes indandione) are widely used in the treatment and prophylaxis of thrombotic disorders. For each patient the dose of these drugs must be adjusted periodically to ensure that an adequate but not excessive degree of anticoagulation is achieved. These adjustments are made on the basis of the results of the prothrombin time or a similar test on the patient's blood. The test, which requires tissue extracts called
thromboplastins, is controlled by the use of calibrated thromboplastins and reference plasmas.

Various types of thromboplastins are prepared commercially and to enable the results of the prothrombin time test to be interpreted, it is important to use standardized reference preparations and to be familiar with the principles of standardization. Attempts at standardization have followed three main approaches: the central provision to clinical laboratories of a single type of thromboplastin (1); the use of stable reference thromboplastins to calibrate the biological activity of other preparations (2); and the use of reference plasmas (3). The second of these was selected to implement international standardization in recommendations published in the twenty-eighth report of the WHO Expert Committee on Biological Standardization (4).

It is possible to effect control of oral anticoagulant therapy at various organizational levels. Thus, a national control system in which standardized reagents are supplied through a national network may be regarded as ideal; but its cost would require justification for each national health authority. Alternatively, hospitals either singly or in regional groups could produce a standardized product for their own use.

In addition, a wide range of commercial reagents are marketed, some on an international basis, and it is essential that their manufacture should be properly controlled so that the final product is correctly calibrated. In such a way, the user will be assured that results of tests with different products and batches will be reproducible and relatable to one another. Standardization was made possible when a way of relating the activity of one preparation of thromboplastin to another using the prothrombin time ratio procedure was devised (4–6). With this procedure the introduction of an international scale for the control of oral anticoagulant therapy is feasible. However the procedure assumes that the prothrombin time ratios, plotted for one thromboplastin against the other, define a straight line passing through point (1, 1). Although this assumption is satisfied in comparing thromboplastins of the same type (so-called like-to-like comparison), there is evidence that it might not hold in comparing international reference preparations of thromboplastins, which are of different tissue source (human, rabbit, and bovine). As a consequence of a straight line not passing through (1, 1), the calibration equations to be dealt with in this report would have to be slightly modified. In the forthcoming EEC/WHO study the assumption will be tested and thereafter the calibration equations, modified if necessary, will be given.
The calibration of a thromboplastin is more precise when comparisons are made between similar preparations of the same species. In order to have a reference point, however, the WHO Expert Committee on Biological Standardization, in its twenty-eighth report (4, p. 14), established a Thromboplastin, Human, Combined, as the International Reference Preparation.

Where a new thromboplastin is prepared for which there is no similar reference preparation, the calibration should be done by an international body against the International Reference Material. In order that more precise calibrations may be made for all thromboplastins however, WHO—in compliance with the recommendations in the twenty-eighth report of the WHO Expert Committee on Biological Standardization (4, p. 51)—has now established International Reference Preparations of Bovine, Combined (coded 68/434) as well as Rabbit, Plain (coded 70/178) Thromboplastins, and it is suggested that laboratories and manufacturers should use these for the calibration of their working reference preparations. These requirements have been formulated, therefore, taking into consideration only the calibration of similar preparations (human with human, rabbit with rabbit, and bovine with bovine).

The purpose of these requirements is to describe in detail the technical methods involved; where appropriate, the particular sections may be adopted by the national control authority or individual laboratory. They have been fitted into the framework adopted in the Requirements for Biological Substances already published by WHO (for list of references see WHO Technical Report Series, No. 638, 1979, Annex 5) and in drafting them account has been taken of the opinions of consultants. In addition, comments have been received from a number of experts (see Acknowledgements, page 199).

1. DEFINITIONS

Thromboplastin: a tissue extract with the ability to accelerate the activation of blood coagulation through the extrinsic pathway, thus bypassing some of the reactions of the intrinsic pathway. Thromboplastins prepared from mammalian tissues contain proteins and phospholipids. A preparation of a thromboplastin consisting of a tissue extract alone is termed “plain”; when the preparation has additional components such as fibrinogen, Factor V, or calcium, it is termed “combined”. Thromboplastins may also be grouped into types, each
made from different tissue sources—e.g., human, bovine, rabbit
brain or lung, or human placenta.

*Thromboplastin, Plain:* a suspension of brain in saline.

*Thromboplastin, Combined:* a suspension of brain in saline, with a
suitable concentration of added bovine fibrinogen, Factor V, and
calcium chloride.

*Prothrombin time:* the clotting time of a plasma sample in the
presence of a preparation of thromboplastin (7).

*Prothrombin time ratio:* the prothrombin time obtained with a
coumarin plasma divided by the prothrombin time with a normal
plasma, using the same thromboplastin in the same test system.

*International Calibration Constant (ICC) (4, p. 47):* the cali-
bration constant obtained when a thromboplastin is compared to (i)
the International Reference Preparations of Thromboplastins, or (ii)
any thromboplastin preparation whose International Calibration Con-
stant has been determined in a statistically valid way (see Appendices 1
and 2).

*International Calibrated Ratio (ICR) (4, p. 48):* a ratio calcu-
lated from the prothrombin time ratio using a thromboplastin with a
known International Calibration Constant. The use of ICRs enables
direct comparisons to be made between all results on patients' plasma
regardless of the laboratory carrying them out or the particular batch
of reagents used (see Appendix 3).

*Plasmas:* plasmas taken with suitable precautions as described in
the prothrombin time method.

Blood from normal subjects and patients should be drawn at approxi-
mately the same time, so that the plasma will be incubated at room
temperature for a similar period of time.

(a) normal plasma: plasma obtained from a healthy person;

(b) coumarin plasma: plasma obtained from a patient stabilized
on oral anticoagulant treatment for six weeks or more, but with no
other interfering haemostatic defect;

(c) fresh plasma: plasma used in the prothrombin time test within
six hours of collection;

(d) control plasma: plasma that has been shown to be stable when
used for its designated purpose.
2. INTERNATIONAL REFERENCE PREPARATIONS
OF THROMBOPLASTINS

The International Reference Preparation of Thromboplastin, Human, Combined was established in 1976 by the WHO Expert Committee on Biological Standardization. It is stored in glass sealed ampoules containing dried thromboplastin prepared from human brain and has an International Calibration Constant of 1.0. The International Reference Preparations of Thromboplastins, Bovine, Combined, and of Rabbit, Plain, were established in 1978 with the assigned International Calibration Constants (4) of 1.0 and 0.6 respectively.

These International Reference Preparations are in the custody of the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Hampstead, London. Samples are distributed free of charge, on request, to national control laboratories. Where there is no national control laboratory they may be supplied to manufacturers. The international reference preparations are intended for the calibration of national reference preparations or of working reference preparations of thromboplastins.

3. PREPARATION OF THROMBOPLASTINS

The method of preparation of thromboplastins shall be such that there is reproducibility from batch to batch and that the preparations are suitable for use in the control of oral anticoagulant treatment.

Thromboplastins of animal origin shall be prepared only from healthy animals. Thromboplastins derived from human tissues shall be prepared only from persons who show no evidence of systemic microbiological infection, localized infection, or tumour of the central nervous system.

The thromboplastins shall comply with the specifications outlined in section 4.

4. TESTS ON THROMBOPLASTINS (8, 9)

Each batch of thromboplastin shall satisfy the following criteria.

4.1 Sensitivity to coumarin-induced defect

The sensitivity to coumarin-induced defect shall be measured by the prothrombin time using normal and coumarin plasmas (see sec-
tion 6.2.1). It shall be similar to the values obtained with other batches of the same thromboplastin prepared by the same manufacturer. The International Calibration Constant shall be not less than 0.3 (10).

4.2 Test for sensitivity to Factor VII defect

The test for sensitivity to Factor VII defect is carried out by the one-stage prothrombin time on plasma from a known Factor-VII-deficient subject. The prothrombin time ratio shall be similar to that obtained with a reference thromboplastin known to be sensitive to Factor VII (10).

4.3 Content of haemoglobin

Attempts should be made to obtain preparations as free as possible from haemoglobin.

4.4 Opacity and sediment volume

The method of manufacture, particularly the method of breaking up the tissue, has a marked effect on the activity, opacity, and sediment volume of the thromboplastin. As photometric equipment may be used to determine clotting time it is advisable that the preparations should be suitable for use in these instruments (see section 6.1.3).

4.5 Freedom from infectious agents

The source of material used for the preparation of thromboplastins will affect the microbiological contaminants likely to be present. The national control authority shall specify the tests used for the detection of such contaminants.

It is desirable but not always possible to prepare sterile thromboplastins. All attempts should be made, however, to use the least contaminated source material and to use a manufacturing procedure that prevents further contamination and growth of organisms during manufacture.

Thromboplastins from human tissue shall be shown to be free from hepatitis B virus (HBV)

Preferably the test should be done by a highly sensitive method, such as radioimmunoassay (RIA), reversed passive haemagglutination (RHIA), or enzyme-linked immunosorbent assay (ELISA).

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4.6 Stability (II)

The method of manufacture of thromboplastin shall be such that the preparations are stable. The stability shall be checked by an accelerated degradation test in which the preparations of freeze-dried thromboplastins shall be shown to maintain activity. The national control authority shall specify the conditions of the test.

It is not possible to incubate thromboplastins for the purposes of this test at temperatures higher than 37°C (2). In general, thromboplastins are stable preparations and should maintain activity as measured by prothrombin time ratio of coumarin plasma for six months when held at 37°C. A thromboplastin losing activity in an accelerated degradation test should not be used. It is not always possible, however, to extrapolate from 37°C to 0°C or −20°C and the stability of the thromboplastins must therefore be determined also for the conditions under which they are stored.

5. CALIBRATION OF THROMBOPLASTINS

Thromboplastins shall be calibrated in terms of the appropriate International Reference Preparation of Thromboplastin and the sensitivity to the coumarin-induced defect shall be defined by the International Calibration Constant obtained in the calibration procedure using a thromboplastin from the same species (4).

The supplies of the International Reference Preparations are limited and it is not possible to use these materials in all tests to calibrate each batch of the many thromboplastins produced by different manufacturers.

Batch-to-batch calibration shall be carried out by comparison with the working reference preparation, which shall be a batch of the same or similar thromboplastin calibrated against the appropriate International Reference Preparation (human to human, bovine to bovine, rabbit to rabbit) of Human Combined, Rabbit Plain, or Bovine Combined.

5.1 The calibration of international reference materials

The calibration of the International Reference Preparations and their future replacements shall be carried out in international collaborative studies.
5.2 The calibration of working reference preparations of thromboplastins

The working reference preparation shall be a batch of a thromboplastin preparation widely used locally. It shall comply with all tests included in section 4. The working reference preparation shall be freeze-dried in ampoules, each of which shall contain enough material for at least 10 tests.

The working reference preparations of thromboplastins of human origin shall be calibrated against the International Reference Preparation of Thromboplastin, Human, Combined (coded NIBSC 67/40); thromboplastins of rabbit brain and rabbit lung shall be calibrated against the International Reference Preparation of Thromboplastin, Rabbit, Plain (coded NIBSC 70/178); and thromboplastin of bovine origin shall be calibrated against the International Reference Preparation of Thromboplastin, Bovine, Combined (coded NIBSC 68/434).

The calibration shall be done by the manufacturers of the thromboplastins.

The calibration procedure (see section 6) may be carried out also by the national control authority or by laboratories designated by it or by a professional body approved by the national control authority.

Unnecessary duplication of calibration should be avoided and where a manufacturer has the expertise in standardization the calibration may be done by the manufacturer alone.

5.3 The batch-to-batch calibration of thromboplastins

The precision of the calibration procedure is greatest when similar materials are compared. For this reason, therefore, the working reference preparation used for batch-to-batch calibration shall be either a comparable batch of the same thromboplastin, or derived from the same tissue of the same species, using a similar manufacturing procedure as that used for the batch with which it is to be compared. The calibration procedure is necessary for both liquid and freeze-dried preparations.

Calibration shall be done by the manufacturers of the thromboplastins.

6. THE CALIBRATION PROCEDURE

The calibration procedure entails the determination of a series of prothrombin times, using normal and coumarin plasmas, with both
the reference and the test thromboplastin (4, 5). The plasmas used are either fresh samples from individual subjects (procedure 1) or freeze-dried or frozen pooled plasmas (procedure 2) from normal subjects and from patients undergoing anticoagulant treatment.

Procedure 1 is recommended for the calibration of the working reference preparation against the appropriate International Reference Preparation, whereas procedure 2 is recommended for the batch-to-batch calibration.

6.1 Procedure 1: Calibration of a working reference preparation of thromboplastin using individual fresh plasma samples

This procedure consists of a set of tests using freshly opened or reconstituted thromboplastins and different individual samples of fresh plasma. The procedure shall be repeated on separate occasions using fresh reagents on each occasion (see section 6.1.4).

The procedure need not be repeated on consecutive days but should be completed as soon as possible. The tests in any one laboratory on any one day should be performed by the same person.

6.1.1 Blood samples

Blood samples from 6 healthy ambulant subjects and 12 patients who have been on oral anticoagulants for at least 6 weeks shall be selected.

Blood shall be obtained by venepuncture avoiding haemolysis and contamination with tissue fluids. It shall be drawn either with a plastic syringe and transferred to a plastic tube, or with other non-contact activation equipment. Nine volumes of blood shall be decalcified with one volume of 100–136 mmol/l trisodium citrate solution. Alternatively, if measured as a final concentration of citrate in plasma, the value shall not exceed 25 mmol/l. The same concentration of citrate shall be used for all the samples in a given calibration.

The procedure recommended by the International Committee for Standardization in Haematology states that the concentration of trisodium citrate (2H₂O) should be 109 mmol/l (7). However, current practice for blood collection includes a range of 100–136 mmol/l citrate, and such plasma may be used satisfactorily with 25 mmol/l calcium chloride as recommended in section 6.1.3.

If vacuum tubes are used for obtaining blood, their lot number should be noted, as there may be a lot-to-lot variation.
The sample shall be centrifuged as soon as received, and the plasma kept undisturbed in a narrow, stoppered, noncontact tube. The sample shall be tested without delay and in any event within six hours. Frozen plasma samples shall not be used.

The plasma need not be taken off the red cell layer.

6.1.2 Reference thromboplastins

The appropriate International Reference Preparations of Thromboplastin (human, rabbit, or bovine) shall be reconstituted as instructed and the contents of the ampoules transferred to a noncontact container in sufficient volume for all tests in a single calibration procedure. Thromboplastins shall be distributed with specific instructions for reconstitution.

6.1.3 The prothrombin time test (7)

The prothrombin time test consists either of mixing equal volumes of citrated plasma, thromboplastin, and calcium chloride solution (25 mmol/l), or of adding a volume of plasma to a volume of thromboplastin that has been premixed with calcium and is therefore available as a single reagent. The time taken for the mixture to clot when maintained between 36.5 °C and 38.5 °C is recorded.

All the reagents shall be brought to the reaction temperature before admixture; thromboplastins must not be preincubated for less than 3 minutes or more than 60 minutes and plasmas shall be preincubated for between 1 and 4 minutes. Calcium chloride solution can be maintained at 36.5–38.5 °C for the duration of the test.

The coagulation endpoint may be detected by a manual technique or with the aid of an automatic endpoint recorder.

The prothrombin times with control plasmas shall be within two standard deviations of the accumulated mean derived from control plasma data from the previous seven or more days.

Records shall be maintained of the lot number of thromboplastins, control plasmas, and vacuum tubes if used. Periodic checks of the temperature of the incubator baths or heating blocks shall be made and recorded.

A suggested protocol for reporting the procedure is shown in Appendix 1.

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6.1.4  Statistical evaluation (12)

To define the calibration constant of a working reference preparation a sufficient number of separate tests shall be carried out to obtain an accuracy of 2% or less, expressed as a coefficient of variation of the calibration constant. It is suggested that the number of normal and patients’ plasmas tested on one working day should be 6 and 12, respectively. In order to reach an accuracy of ≤ 2% the total number needed might be as high as 30 and 120, respectively. Hence, testing on 5 different days may be necessary.

6.2  Procedure 2: Batch-to-batch calibration of thromboplastins using pooled plasmas

6.2.1  Requirements for pooled plasmas

6.2.1.1  Properties of pooled normal plasma. Plasma shall be obtained from healthy adults and shall comply with the WHO Requirements for the Collection, Processing and Quality Control of Human Blood and Blood Products (13, pp. 28–93).

The pooled normal plasma should be obtained from at least 20 different donors.

The final preparation shall be platelet-poor plasma, which has been freeze-dried or frozen (at −30°C or below) in suitable containers. After reconstitution or thawing, the pH shall not exceed 7.8, and the preparation shall not show any shortening or prolongation of clotting times for at least 2 hours when held at ambient temperature.

The stability of freeze-dried normal plasma should be checked by accelerated degradation tests. Such plasma should not show a prolongation of prothrombin time of over 5% after 4 weeks at 37°C.

The International Calibrated Ratio of the pooled normal plasma shall be stated and shall be between 0.8 and 1.2.

The Factor V content shall be between 70% and 140% of that of average fresh normal plasma.

To permit the determination of the end-point by photoelectric coagulometers, the turbidity at 320 nm shall be less than 2.0.

In order to obtain the theoretically calculated values of citrate it is assumed that nine volumes of blood are decalcified with one volume of 100–136 mmol/l trisodium citrate (2H2O) solution. The same concentration of citrate shall be used for all the samples in a given calibration. The haematocrit value is about 0.40.
6.2.1.2 Properties of pooled coumarin plasma (14, 15). Pooled coumarin plasma is obtained (as described in section 6.1.1) from patients who have been “stabilized” on long-term oral anticoagulants for at least six weeks.

In order to ensure that a patient has become “stabilized”, the clotting times of two samples taken no longer than 14 days apart should not differ by more than 20% in terms of the ICR.

In some countries, the plasma is obtained either from a single donation or from multiple donations from the same donor bled on different occasions and pooled together, or from donations from different donors with similar levels of anticoagulation. In other countries, however, the pooled plasma is collected from at least 30 donors.

Plasma shall not be obtained from donors with a history of jaundice or from those with plasma lipid abnormalities.

The collection of plasma, the properties of the final preparation, and the stability of the freeze-dried pools are described in section 6.2.1.1.

The International Calibrated Ratio of the pooled plasma shall be stated. At least two different plasma pools having an International Calibrated Ratio between 2.0 and 4.0 are necessary for the calibration procedure.

The Factor V content, turbidity, and citrate concentration shall comply with the requirements for normal plasma (see section 6.2.1.1).

6.2.1.3 Freedom from infectious agents. The plasma shall be shown to be free from hepatitis B virus (HBV).

Preferably the test should be done by a highly sensitive method, such as radioimmunoassay (RIA), reversed passive haemagglutination (RPHA), or enzyme-linked immunosorbent assay (ELISA).

It is preferable for plasma pools to be sterile.

6.2.2 The test

The test shall be carried out by the same procedure as described for procedure 1 (see section 6.1.3) and an example of the protocol for the recording of the results is given in Appendix 2.

The freeze-dried plasma samples shall be reconstituted immediately prior to testing and the volume of each plasma required for a single calibration procedure pooled in a noncontact container (7, 12). Frozen or reconstituted freeze-dried plasma shall not be centrifuged, and unused reconstituted or thawed material shall be discarded after two hours.
6.2.3 Statistical evaluation (12)

To define the calibration constant of a batch of thromboplastin a sufficient number of separate tests shall be carried out to obtain a reproducibility of 2% and an accuracy of 3%, expressed as a coefficient of variation of the calibration constant.

7. ADOPTION OF THE USE OF CALIBRATED THROMBOPLASTINS IN CLINICAL PRACTICE

It is possible to express all results of prothrombin times on a common scale—i.e., International Calibrated Ratio (ICR)—if the International Calibration Constant of the thromboplastin used is known.

The following formula is used: \( \text{ICR} = 1 + \frac{(R-1)}{\text{ICC}} \), where \( R \) is the prothrombin time ratio (patient time divided by normal time) and ICC is the International Calibration Constant of the thromboplastin used. The ICR is thus the patient’s corrected prothrombin time ratio, enabling comparisons to be made between the results obtained using different thromboplastins (see Appendix 3).

All medical staff and health auxiliaries involved in controlling anticoagulant treatment in patients should be encouraged to use the ICR. It is unrealistic, however, to expect physicians, laboratory technologists, and patients to adopt a new way of expressing results immediately. It is suggested, therefore, that the results should be reported both in the usual method and in ICRs for the time being. In order to facilitate the use of ICRs, however, the manufacturers should produce a table, an example of which is given in Appendix 4, which should accompany each batch of thromboplastin.

8. LABELLING

8.1 Thromboplastins

The label on the containers of thromboplastins shall show:
— the name of the product;
— the name of the manufacturer;
— the number of the final lot;
— the source of material; and
— the International Calibration Constant.
The label on the package shall, in addition to the information shown on the label on the container, show:

— the nature and amount of any preservative, stabilizer, or added substance;
— the conditions of storage;
— the expiry date; and
— the stability of the reconstituted thromboplastin.

The leaflet shall include instructions for reconstitution.

The package shall include a table of International Calibrated Ratios for the specific batch of thromboplastin (see Appendix 4).

8.2 Plasmas

The label on the containers of plasmas shall show:

— the name of the product;
— the name of the manufacturer;
— the number of the final lot;
— the type of material; and
— the International Calibrated Ratio.

The label on the package shall, in addition to the information shown on the label on the container, show:

— the prothrombin times and the appropriate International Calibrated Ratio;
— the conditions of storage;
— the expiry date; and
— the stability of the reconstituted or thawed plasma.

The leaflet shall include instructions for reconstitution and shall specify the conditions of use.

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ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice and for supplying additional data relevant to these Requirements:

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REFERENCES


**Appendix 1**

**SUGGESTED METHOD FOR REPORTING THE DATA FOR THE CALIBRATION OF A WORKING REFERENCE PREPARATION OF THROMBOPLASTIN AGAINST THE INTERNATIONAL REFERENCE PREPARATION**

**Date:** 12.7.77

**Thromboplastins:**

1. Rabbit brain thromboplastin working reference preparation
2. International Reference Preparation of Thromboplastin Rabbit Brain, 70/178

**End-point recording:** coagulometer

**Time started:** 10.15

**Time finished:** 11.00

200
Table 1. Suggested protocol for the recording of clotting times in the calibration of a working reference preparation of rabbit thromboplastin (prothrombin time)

<table>
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<tr>
<th>Order of testing</th>
<th>Plasma No.</th>
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<th>Order of testing</th>
<th>Plasma No.</th>
<th>Coumarin plasma clotting time (seconds)</th>
<th>Order of testing</th>
<th>Plasma No.</th>
<th>Normal plasma clotting time (seconds)</th>
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Mean time: 12.2 s
Mean ratio: 1.90
Mean time: 14.4 s
Mean ratio: 2.05

*Each individual coumarin plasma clotting time is divided by the corresponding mean of the normal plasma clotting times to obtain individual ratios for plotting in Fig. 1. If the formula on page 202 is used, it is quicker to obtain the mean ratio by dividing the means of the normal plasma clotting time into the means of the corresponding coumarin plasma times; individual ratios need not then be calculated.
Calculations

1. International Calibration Constant (ICC) is obtained by plotting the prothrombin time ratios using the two thromboplastins as shown in Fig. 1, fitting a straight line by eye to pass through the origin and measuring the slope; or the slope can be calculated from the following formula:

\[ \text{ICC}_{\text{WRP}} = \frac{\text{ICC}_{\text{IRP}} (R_{\text{WRP}} - 1)}{(R_{\text{IRP}} - 1)} \]

where

- \( \text{ICC}_{\text{WRP}} \) = the ICC of the working reference preparation
- \( \text{ICC}_{\text{IRP}} \) = the ICC of the International Reference Preparation (rabbit = 0.6)
- \( R_{\text{WRP}} \) = the mean ratio for the working reference preparation
- \( R_{\text{IRP}} \) = the mean ratio for the International Reference Preparation

For example, for a rabbit thromboplastin, using the data from Table 1, the ICC for a working reference preparation is:

\[ \text{ICC}_{\text{WRP}} = \frac{0.6 (1.90 - 1)}{(2.05 - 1)} = 0.51 \]

Fig. 1. Plot of prothrombin time ratios for determination of the International Calibration Constant
Appendix 2

SUGGESTED METHOD FOR REPORTING THE DATA
ON THE BATCH-TO-BATCH CALIBRATION OF
THROMBOPLASTIN

Date: 11.8.77 Instrument temperature 37.2 °C
Thromboplastins: 1. Bovine brain thromboplastin working reference preparation, and
2. Batch of bovine brain, thromboplastin
End-point recording: coagulometer
Pooled coumarin plasmas, lots 683, 684, 685, 686, 687
Pooled normal plasma: lot No. 2
Time started: 11.02 Time finished: 11.25

Calculation

The International Calibration Constant of the batch

$$ICC_b = \frac{ICC_{\text{WRP}} (R_b - 1)}{(R_{\text{WRP}} - 1)}$$

where

- ICCb = the ICC of the batch of thromboplastin
- ICCWRP = the ICC of the working reference preparation (bovine = 0.98)
- Rb = the mean ratio for the batch of thromboplastin
- RWRP = the mean ratio for the working reference preparation

For a batch of bovine thromboplastin, therefore, using the data from Table 2 (see page 204), the ICC for this batch of bovine brain thromboplastin is:

$$\text{International Calibration Constant (ICC)} = \frac{0.98 (2.79 - 1)}{(2.79 - 1)} = 0.98$$

Appendix 3

CALCULATION OF INTERNATIONAL CALIBRATED RATIO (ICR) FOR A GIVEN PATIENT'S CLOTTING TIME

Example:

Thromboplastin: rabbit, International Calibration Constant = 0.51
Patient’s prothrombin time = 28 seconds
Normal prothrombin time = 12 seconds
Therefore, prothrombin time ratio = 2.33

To calculate the ICR the following formula is used: $1 + \frac{(R-1)}{(ICC)}$

where $R =$ prothrombin time ratio

$$ICC = \text{International Calibration Constant}$$

Thus the ICR is $1 + \frac{2.33-1}{0.51} = 3.61$
Table 2. Suggested protocol for the recording of clotting times in batch-to-batch calibration of thromboplastin (thrombotest)

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Appendix 4

INTERNATIONAL CALIBRATED RATIOS OF PATIENTS' PLASMA USING A THROMBOPLASTIN WITH AN INTERNATIONAL CALIBRATION CONSTANT OF 0.51

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Therapeutic range

N = average normal plasma prothrombin time in seconds.
P = patient’s plasma prothrombin time in seconds.
ICR = International Calibrated Ratio using a batch of thromboplastin with a calibration constant of 0.51.
### Annex 8

**REQUIREMENTS FOR IMMUNOASSAY KITS**

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### INTRODUCTION

The use of immunoassay kits for the estimation of a great variety of analytes in body fluids is now established on a very wide scale. Many manufacturers and individual laboratories are producing kits that make a very significant contribution to health care throughout
the world. Nevertheless, there may be kits that do not give reliable and uniform results. Several national and international bodies are now preparing independent specifications for the regulation of diagnostic kits.

It is recognized that problems arising from diversities in such regulations could be avoided by establishing a set of international requirements and guidelines for kits and their components. Recommended criteria for the assessment of radioimmunoassay reagents appeared in the twenty-sixth report of the WHO Expert Committee on Biological Standardization (I, p. 29); the present Requirements are intended to replace them. These Requirements and the accompanying Guidelines should apply to all kits used in health care, whether they are assembled by manufacturers or by national or independent laboratories. It is emphasized, however, that although the quality and quantity of each component plays an integral part in the performance of the kit, the usefulness of a component can be assessed only by measuring the performance of the whole kit when it is used according to the manufacturer's instructions.

The assembly of kits is a complex procedure demanding considerable experience, expertise, and financial investment. Any national or international body or individual laboratory contemplating the production of kits should carry out a careful cost–benefit analysis to determine whether the necessary expertise is available and the investment justifiable. An important consideration in such an analysis is the possible variability of results within or between batches or lots. In any event, the test results should correlate with those obtained with validated kits and by other methods, on the basis of which analyte concentration ranges considered normal or abnormal have been proposed for a particular body fluid. In clinical practice, the safety of the patient and the effectiveness of treatment may well depend as much on the quality of a kit and its method of use as on the quality and quantity of a drug used in treatment.

GENERAL CONSIDERATIONS

An assay kit is defined as a set of reagents and materials intended for the estimation in vitro of a specified analyte to a stated degree of precision when used according to the manufacturer's instructions. The purpose of these requirements is to help ensure that enough relevant information is provided to enable a national control authority to
decide whether a kit is suitable for the purpose stated by the manufacturer.

To make an adequate assessment of each kit, or kit component, the national control authority should consider the data indicated in these Requirements and Guidelines in order to determine the extent to which they have been followed. Even after adequate assessment and the release of the product by the national control authority, the decision as to the acceptability of the kit for a particular purpose is the responsibility of the user, who should base it on the actual performance of the kit for the required purpose.

Manufacturers should provide sufficient information to enable the performance of their kits to be assessed according to the criteria recommended in Part C. Such information could also facilitate the comparison of products from different manufacturers. In addition, the national control authority should ascertain that the routine measures taken by the manufacturer to ensure their quality are adequate.

A kit should be evaluated as a whole on the basis of its performance when used according to the manufacturer’s instructions and not just according to criteria for individual reagents. Therefore all the reagents used for the assay of a particular analyte should be assessed together to ensure that the precision and specificity are as stated by the manufacturer.

The specificity of the assay system is its ability to estimate solely the type and kind of analyte it is intended to measure. It should therefore be determined for the particular molecular form of the analyte and the nature of the test specimen it is intended to assay, and the results should be expressed either in quantitative or in “Yes/No” terms. Consequently, for each analyte assay system, there is a need for appropriately characterized reference preparations that will permit the specificity of a kit to be checked and will ensure that the estimates are not affected by other substances that may be present in the test samples. For example, the reference materials for peptide hormones may consist of highly purified samples of various intact hormones and pure preparations of various related materials, such as the hormone precursor or a subunit (2, pp. 7–10); for analytes available in pure chemical form, such as steroids and drugs, samples of various related substances known to be chemically similar would be used; for hepatitis B surface antigen (HBsAg) a panel of sera for the hepatitis antigen subtypes would be used (International Reference Reagents consisting of a panel of such sera were established in 1978 (3, p. 29)); for tumour marker substances, various normal serum
proteins, normal cell constituents, and related substances from neoplasia of various types would be used. The national control authority should supply, or approve, such reference materials for use in assessing specificity.

The calibration of kits for the estimation of different types of analyte presents different problems for each type. Kits intended for the assay of pure chemicals, such as steroids and certain drugs, should be calibrated in terms of samples of the particular chemical in the purest attested form available. Kits intended for the assay of peptides, proteins, and other biological substances that cannot be characterized entirely by chemical and physical means alone should be calibrated in terms of attested biological reference materials such as the appropriate International Reference Preparation. National control authorities should supply, or approve, reference materials used for the calibration of kits. Guidelines for the preparation, handling, and characterization of such reference materials are available (4, Annex 4).

A number of different methods exist for the statistical calculation of assay results. This poses a problem only when significantly dissimilar estimates can be shown to depend on the statistical procedures used to calculate them. In any treatment of data, it is preferable for curve fittings to be done by an objective statistical method with validated computerized data processing.

Attention is drawn to certain recommendations of the WHO Expert Committee on Biological Standardization concerning the manner of expressing the results of immunoassays of hormones (2, pp. 7–10). When the results of such assays are given, the qualification “by immunoassay” should be added; in addition, if the results are given in units, it is essential that the reference material on which the unit was based should also be indicated. In all cases, if any preparation (e.g., an international, or other widely recognized, reference material) has been used to calibrate the reference material in the assay system, it should be identified. Problems that may arise in the calibration or replacement of reference materials in different assay systems have also been reviewed by the Expert Committee (1, pp. 45–48).

When results are given in terms of mass concentration or substance concentration, SI units\(^1\) should be employed. For ease of comprehen-

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\(^1\) The use in medicine of the Système international d’Unités (SI) developed by the Conference générale des Poids et Mesures was endorsed by a resolution (WHA 30.39) of the Thirty-eighth World Health Assembly in 1977. A succinct, simple and authoritative account of the use of SI units—*The SI for the Health Professions*, Geneva, World Health Organization, 1977—is now available.
sion in laboratories where these units are not used, appropriate conversion factors should be provided, but the importance of using SI units should be stressed.

While normally the radiation hazard involved in the use of radioimmunoassay kits is negligible, all work with radioactive materials should be carried out in conformity with national legislation and accepted codes of practice for protection against radiation hazards. A set of recommendations on precautions to be taken in dealing with potentially infective materials is being prepared by WHO. It includes directions for the proper disposal of waste material that might constitute a public health hazard.

A. REQUIREMENTS FOR THE MANUFACTURE AND ASSEMBLY OF IMMUNOASSAY KITS AND KIT COMPONENTS

A kit shall comprise a number of matched components, including the tracer-labelled ligand (or tracer ligand), the binding protein (or binder), the calibration material (or calibrator), and such other items as separation material and buffer solutions.

A kit may sometimes include key accessory items—e.g., membrane filters—that are necessary for the optimum functioning of the assay system.

As the performance characteristics of a kit are governed by the interrelationship between the components and the procedure used for carrying out the assay, and deviation from the recommended procedure may influence the performance characteristics and render the assay result invalid and unsuitable for its intended purpose, the kit shall be used according to the instructions accompanying it.

While the characteristics of individual components can be specified to some extent, the ultimate suitability of each component can be ascertained only when it is tested with all the other components and according to the full procedure that will be used with the kit in its final form.

1. TRACER-LABELLED MATERIALS

All tracer-labelled materials shall be prepared from the most suitable ligands available, as determined by tests approved by the national control authority.

1.1 Tracer-labelled ligand

The tracer-labelled ligand shall be prepared from the ligand or an appropriate chemical derivative of the highest available purity.

The use of pure materials in the assay reduces nonspecificity due to extraneous cross-reacting contaminants. Often the ligand requires chemical modification before or during the labelling process and the procedures used are determined by the type of label—e.g., radionuclide, enzyme, fluorophor, or red blood cell.

The purity and suitability of the ligand shall be assessed both before and after labelling, using a variety of physicochemical and immunochemical procedures approved by the national control authority.

The tracer ligand is usually required to have a high degree of purity in order to produce an assay system with the required characteristics. In some instances, however, satisfactory assay systems can be developed with tracer ligands of only moderate purity.

The performance of the tracer ligand in the final kit is particularly important. The tracer ligand selected shall possess sufficient stability to maintain performance of appropriate quality over a specified acceptable period of time.

1.2 Tracer-labelled binding protein (tracer binder)

The tracer-labelled binding protein used shall be approved by the national control authority.

Up to now, in almost every case in which a tracer-labelled binding protein has been used it was derived from an antibody preparation—e.g., a purified immunoglobulin fraction, or antibodies obtained by affinity chromatography. In future, antibodies from monoclonal hybridomas may become available. The selection of a preparation often depends on the titre of the original antiserum; a high titre and a low degree of cross-reaction with potentially interfering substances indicate potential suitability.

The degree of purity of the final labelled antibody may vary considerably depending on the analyte being assayed, the sensitivity required, and the design of the assay system.
Certain kits require a labelled antibody preparation that has been purified by affinity absorption until more than 50% of it will bind specifically to excess solid-phase antigen. Because of the nature of the procedure (number of wash steps, extremely low nonspecific binding, etc.) other kits may function satisfactorily with labelled antibody of a relatively low specific purity, provided it is free of uncoupled label.

2. ANTISERA AND OTHER BINDING SUBSTANCES

The immunogens used for the production of specific antibodies shall be the purest preparations available. The method of production shall be approved by the national control authority.

2.1 Characteristics of the antisera

The antiserum used in a kit shall be characterized by tests capable of identifying it so that there will not be differences in performance from batch to batch.

The characteristics required for an antiserum or other binding protein used in a kit will depend on a variety of its properties, other constituents of the particular assay system in which it is used, the separation procedure, and the physicochemical conditions in the assay system, such as the buffer and the type of test specimen.

In the case of substances of large molecular weight to be used as immunogens, the preparation should preferably be highly purified in order to avoid immunization with other closely related substances such as metabolic products of the analyte.

Alternatively, it may be possible to prepare monoclonal antibodies of high and constant specificity following exposure to an immunogen (even if impure) and an appropriate lymphocyte clone selected for the production of suitable antibodies.

In all cases, if an antiserum has been shown to be suitable for a given assay kit the nature of the immunogen used is irrelevant.

2.2 Quality of the antisera

The quality of an antiserum shall be evaluated, using the whole assay kit.

An antiserum that is satisfactory when used with a certain tracer or separation system may not be suitable for use with another.
Adequate precautions should be taken to prevent gross microbial contamination of antisera and preparations of binding substances during manufacture; in many cases, suitable preservatives—e.g., bacteriostatic or bacteriocidal agents—may be added to prevent product deterioration during storage, after reconstitution, and during subsequent use.

3. REAGENTS FOR SEPARATION OF BOUND FRACTION AND FREE FRACTION

Various techniques are used for the separation of bound and free antigens. The separation system chosen shall have been shown to work effectively with the kit when it is employed according to the manufacturer’s instructions.

It is desirable that the separation system should be as little influenced as possible by such variables as temperature, time, and pH, or by the nature of the biological fluid concerned. Furthermore, it should be relatively simple to operate.

4. REFERENCE SERA

The performance of the kit shall be checked batch by batch with the aid of reference sera.

In the control of kit manufacture it has been found extremely helpful for the manufacturer to maintain a collection of reference sera with which to check the performance characteristics of the kit. Such sera normally consist of samples containing various concentrations—e.g., high, medium, and low—of an endogenous analyte; sera to which measured quantities of analyte or calibrator have been added; sera containing no analyte (if possible, without artificial removal of the analyte); and one or more samples of serum from patients with recognized pathology that might be used in certain cases in association with the assay system—e.g., serum from patients with acromegaly in the case of kits intended for assay of growth hormone. Such sera are useful for the qualitative assessment of specificity and parallelism, but they may also be used for checking the calibration of kits; for this purpose, they may be assigned consensus values of analyte concentration derived from repeated independent assays against the calibrator.

The reference sera shall be stored under conditions that will ensure maximum stability—e.g., in the form of aliquots in suitable sealed containers at, or below, 0°C, or freeze-dried.
National authorities shall provide or approve the reference sera for the calibration of the sera supplied by the kit manufacturers and recommend appropriate conditions of storage.

Analyte values (if assigned) should be provided. In the case of certain analytes, it may be desirable to provide one or more reference sera for national or international use. For ease of distribution, it is expedient to freeze-dry these sera in ampoules, which can then be sealed, but it is essential to ascertain that the analyte has not been irreversibly altered by the desiccation process. To obtain consensus values for such sera, it is advisable to include samples of them in collaborative studies such as those carried out in connexion with the setting-up of international or national standards—e.g., the preparation of postmenopausal plasma, NIBSC No. 69/178, included in the international collaborative study of the International Reference Preparation of Human Pituitary FSH and LH, for bioassay.

The reference sera referred to here should not be confused with quality control sera, which are samples of sera included routinely in all assay series for purposes of within-laboratory quality control (internal quality control), or those distributed on a regional, national, or international basis for between-laboratory quality control (external quality control).

5. REFERENCE MATERIALS

Reference materials used in the calibration of the kit shall include international and national reference preparations, the manufacturer’s house standard, and the calibrator(s) in each kit.

The calibrator in the kit should contain the substance in the same molecular form as the analyte in the test specimen, or resembling it insofar as its behaviour in the kit assay system is the same over the dilution range used—i.e., it shows parallelism (using a suitable statistical transformation) over the working range of analyte concentration for which the kit is intended to be used.

Each sample of a specific lot of calibrator with an assigned value, in the form supplied in each kit, shall be identical.

The stability of the calibrator should be estimated by accelerated degradation studies or other acceptable methods and shall be shown to be adequate for the temperature conditions likely to be encountered by the kit.
The calibrator in the kit shall be calibrated in its final form by comparisons with the primary (e.g., international) reference material where appropriate.

For this purpose, it may be necessary to use dilutions of such material in suitable analyte-free biological fluids (e.g., serum) which the kit is intended to assay. Calibration of the house standard and of the kit calibrator should be based on the results of several independent assays—e.g., starting with fresh dilution procedures—so that the value assigned is precise.

In the case of peptides, proteins, and other substances that cannot be completely characterized by chemical and physical means alone, International Biological Standards and Reference Preparations shall be used for calibration; these and International Reference Reagents shall also be used also for the assessment of the specificity of the kit assay system for the intended analyte.

A detailed description of the selection, preparation, ampouling, characterization, and calibration of international and national reference materials is given in the twenty-ninth report of the WHO Expert Committee on Biological Standardization (4, Annex 4).

The successive reports of the Expert Committee (published approximately annually) contain up-to-date information on the establishment of international biological reference materials. Technical information concerning the nature, characterization, and calibration of each international reference material is normally sent with each distribution of ampoules, and further information may be obtained from the international laboratory responsible for its distribution.

B. REQUIREMENTS FOR LABELLING KITS, COMPONENTS, AND PACKAGE INSERTS

All components of a kit and the container of the kit shall be clearly identified by labels. The information given on the label on the container or the label on the package shall be approved by the national control authority. The labels on the containers shall be attached in such a manner that the contents can be seen and that the label is not easily detached. Any abbreviations used shall be approved by the national control authority.
1. LABELS ON CONTAINERS

The labels on the containers of the components, including reference materials, shall show at least the following.

— The name of the product.

   The name should be the International Nonproprietary Name, where appropriate, but a proprietary name may also be included.

— The name of the manufacturer.

   This should be the registered name of the manufacturer or licence holder. Sufficient information should be included to allow a user of the product to make direct inquiries.

— The lot number.

   Only from this number or code can the manufacturer identify the manufacturing history of the components.

— Storage and expiry date.

   The expiry date should be based on data obtained under specified storage conditions. These conditions should be stated along with the expiry date. Should the expiry date and storage conditions be different after reconstitution, this should be stated.

— A statement whether the component may or may not be used with other lots of the same kit or other kits.

— A caution drawing attention to any hazards.

   If any component may be confused with a product administered to humans, a cautionary statement such as “for laboratory use only” must be used. If a hazard is associated with the product, cautionary words such as “CAUTION” or “RADIOACTIVE” should be used, together with the appropriate internationally adopted symbol.

   In addition, the label of a component that is an antiserum, shall indicate the animal species in which it was raised or whether it was produced in vitro in normal, transformed, or cancer cell lines.

2. LABELS ON PACKAGES

The labels on the packages shall, in addition to the information shown on the labels on the containers, indicate at least the following.
— The purpose for which the assay is intended.

For example, "Kit for estimation of free thyroxine in blood serum/plasma". Ambiguity should be avoided when a kit or material may have several uses, each requiring different concentrations or preparations—e.g., "Kit for estimation of human chorionic gonadotrophin in blood serum/plasma or urine"; "Kit for estimation of free or total thyroxine in blood serum/plasma"; "Kit for estimation of human placental lactogen in blood serum/plasma in the first, second, or third trimester of pregnancy".

— The components, including the number of vials, ampoules, or bottles in each container in the kit, and the number of assay tubes for which the quantities of reagents provided are considered sufficient for particular concentrations of analyte.

3. INFORMATION GIVEN ON THE PACKAGE INSERT OR BROCHURE

Every kit shall contain a package insert or brochure that fully describes the kit, including the calibrators, and gives precise instructions on its use. The information shall repeat the information given on the label on the package and, in addition, shall indicate at least the following.

— The principle of the test.

A concise explanation of the type (e.g., radioimmunoassay) and principle of the test should be given, accompanied by appropriate references to the literature.

— Precautions.

A description of known hazards and necessary precautions must be given. It is possible to give much more detail on the package insert than on the label on the package.

— Suitability for use.

If appropriate and if the information is available, there should be a statement of the physical, biological, or chemical indications of instability or deterioration. Where applicable, descriptions of tests that the user may apply to assure satisfactory performance should be included.

— Reagents.

The names and contents of reagents in the kit shall be stated when necessary for the proper performance of the procedure, as shall the quantity, proportion, or concentration of each ingredient, the generic
source or sources, potency, specificity, avidity, sensitivity, or other specifications as appropriate.

For a product derived from biological material, the generic source or, if relevant, the microbial group, type, and strain should be given.

There should be a statement to the effect that the calibrator or kit has been calibrated against a specified recognized reference preparation and the estimated value should be given.

— Other materials.

The nature and characteristics of additional materials shall be stated.

The characteristics of material such as a buffer, preservative, or stabilizer could affect the proper performance of the test or influence the results.

— List of materials supplied by the manufacturer.

If should be stated in particular whether any components are interchangeable with those of other kits and, if so, under what circumstances.

— List of materials required but not supplied by manufacturer.

Materials not supplied by the manufacturer but required for the performance of the test must be listed. Indications of the purity (e.g., analytical grade) of the materials, procedures for diluting or mixing, and other pertinent information permitting assessment of proper performance.

— Equipment.

The specifications of any special equipment required should be given—e.g., type of rotor or gravitational force (g) needed for centrifugation.

— Specimens.

Where applicable, mention should be made of:

(i) special precautions regarding specimen collection, including special requirements regarding the preparation of the patient—e.g., for estimates of remin, the time of day, and the position of the patient;

(ii) additives—e.g., preservatives necessary to maintain the integrity of the specimen;

(iii) known interfering substances—e.g., particular anticoagulants; and

(iv) recommended storage and handling or shipping instructions for the protection and maintenance of the stability of the specimen.
— Instructions for use.

Every detail of the operations required to achieve correct results must be stated clearly in sequence. It is helpful to have working instructions presented in a durable form suitable for continued use on the laboratory bench. Guidance should be given on:
(i) precise experimental conditions that must be met—e.g., pH, temperature, incubation times for specific steps in separation techniques;
(ii) the calculation of results, including an explanation of each factor and step in the calculation (if appropriate, an illustrative sample calculation should be given, including a typical dose-response curve); and
(iii) procedural techniques that may assist the user to perform the test more effectively—e.g., checking purity of water, pipette calibration, pouring supernate from precipitate compared with aspiration, and proper sequence of addition of reagents.

— Calibration procedures.

Calibration procedures, including the preparation of the calibration material as well as the construction of a dose-response curve with the calibration material, must be described.

— Precautions during the conduct of the test.

Any precautions that should be taken during the test in addition to those applicable to the components should be stated. These will relate to radiation, contamination with microbial materials, disposal of waste material, chemical toxicity, etc.

— Validity.

The following information should be included:
(i) the advantages, limitations, precision, bias, etc., of the method;
(ii) for clinical application, expected results and details of how the data were derived, identifying the populations used, both for normal and pathological states; and
(iii) a full description of the use of the reference materials and calibrators.

The results should be expressed in units in common use; where applicable, SI units must be used. The confidence limits pertaining to any results reported and the method of calculation should be stated.
C. GUIDELINES FOR ASSESSING THE PERFORMANCE OF IMMUNOASSAY KITS FOR HEALTH CARE

1. GENERAL

These guidelines are intended to cover the information which manufacturers shall have available for the national control authority. They are not entirely appropriate for the following situations:

— use of the kit by a manufacturer for additional measures for its assessment other than those specified in the Requirements;
— the practical assessment of kits by national control laboratories; and
— the assessment of kits by individual users (this important subject will be dealt with in another WHO document now in preparation).

While the quality of the individual components of a kit is clearly important and will govern decisions during the assembly and manufacture of kits, the performance of the whole kit shall be the ultimate criterion for the quality of the kit and undue attention to the quality of the separate reagents may be irrelevant and counterproductive.

In all instances involving the processing of data from dose-response curves or the statistical assessment of errors, details of the calculation procedures shall be made available by the manufacturer.

In principle, the assessment of the performance of immunoassay kits does not differ from that of many other diagnostic kits in clinical chemistry—e.g., those for urea. In practice, particular difficulties arise in the establishment of analytical validity (specificity and bias) and in the maintenance of assay-to-assay reproducibility.

It is recognized that a given manufacturer may produce various types of kits, each for a different analyte or, sometimes, for the measurement of more than one analyte.

— When a manufacturer supplies a kit providing results that may be used to assist clinical diagnosis, the intended clinical application must be stated, and the manufacturer is required to obtain evidence to support the statement.
— If, for a given clinical condition, no data are presented, it is implied that the manufacturer (at that time) does not offer the kit as suitable for assays relating to that condition, or has not com-
pleted validation of the kit for the assays in question. In such a case, should a user choose to use the kit for such assays, he would do so on his own responsibility.

— A manufacturer may also produce a kit for an assay (of a stated analyte) having no claim to clinical usefulness—for example, for the assay of a newly discovered compound whose clinical application is as yet unassessed. In this case it is the concern of the control authority to ensure that, while the manufacturer may not make or be able to substantiate any claim of clinical usefulness, such kits should be available for investigatory use, provided that the same performance characteristics as for in vitro diagnostic kits are fulfilled, apart from justification of clinical application.

The production of reliable kits for a newly identified analyte can be of great importance in the evaluation of potential diagnostic application(s).

2. ASSESSMENT OF RANDOM ERRORS (PRECISION) OF IMMUNOASSAY KITS

2.1 Within-assay series precision and detection limit

The reproducibility (precision) of replicate measurements in a given dose range in an assay will determine the reliable working range of that system. In immunoassays some of the errors leading to poor precision are a feature of the assay design, but others (e.g., poor pipetting, counting errors) are largely beyond the manufacturer's control. An important factor in kit design is that it gives maximum precision at analyte concentrations of maximum clinical interest, although it is appreciated that, with the use of certain provocative tests such as the measurement of insulin following a glucose load, analyte concentrations may be so high that it may be necessary to recommend appropriate dilutions of the test sample.

The precision of repeat estimates within one assay series of specimens covering the clinically relevant analyte concentrations of the dose-response curve shall be indicated. This "precision profile" shows the working range of the assay. The detection limit of the assay is the least amount of analyte that can be differentiated from zero with a designated probability. Particular attention shall be given to the ranges of analyte concentration considered normal and those at
which clinical decisions will be made. At this stage, data obtained by
the manufacturers are used, since data from user laboratories could
be misleading. Details regarding the number of replicate determi-
nations and the number of occasions upon which the precision was
measured (perhaps including different batches or filling lots of critical
reagents) shall be given.

2.2 Between-assay series precision (reproducibility)

Between-assay series precision can be affected by many factors,
such as change of operator, change of a component, or even the use of
a different filling lot of a component. The extent to which an assay is
insensitive to such factors is called ruggedness.

The manufacturer shall provide details of the reproducibility of
the performance characterized and inform the control authority of the
method used to assess it. As a guideline, between-assay variation
shall be assessed at a minimum of three dose levels covering the range
of analyte concentrations likely to be encountered in clinical applica-
tions, especially those at which clinical decisions may be made. As-
sessments of variation should be made in respect of the shelf-life of
lots and batches of critical reagents, and from lot to lot and from
batch to batch of the complete kit.

3. ASSESSMENT OF SYSTEMATIC ERROR (BIAS) OF
IMMUNOASSAY KITS

Evaluation of the validity of the performance of immunoassay kits
involves certain matters of principle and practice. For many analytes
(e.g., peptide hormones) there may be no ascertainable “true value”
in strict analytical terms against which the bias of a kit can be assessed.
Even if there is such a value, it may not be clear how it should be
assigned. For some analytes with a well-defined chemical structure,
so-called reference methods may be available—e.g., mass spectro-
metry, or isotope dilution mass fragmentography for steroids and
other compounds of small molecular weight. But “reference meth-
ods” for the estimation of analyte concentration in biological fluids
should be used with caution by control authorities, as at present there
are few data about the precision, reproducibility, and bias of the
methods in use.

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Many other procedures are used to evaluate the specificity of a kit assay system and these shall be agreed with the control authority. The following are useful criteria but each in itself is insufficient to demonstrate validity.

— *Parallelism* of estimates of analyte and calibrator diluted in the biological fluid for which the kit is intended.
— *Recovery* of known amounts of reference material added to such biological fluid.
— *Comparison with other methods*—e.g., after further purification of the specimen by chromatography.
— *Agreement of values (consensus) with those obtained by other methods* or other kits for specimens obtained in well-defined physiological or pathological situations.

4. **SPECIFICITY**

Specificity of the binding protein indicates the potential specificity of the complete kit but is not enough in itself to ensure that the kit has sufficient specificity. In certain circumstances, the effect of certain factors—e.g., the nature of the specimen of biological fluid and the effect of interfering drugs and metabolites—may need to be determined. It is clear that all possible factors that could influence kit usage cannot be investigated initially, but information comes with experience in using the kit. Manufacturers should collect evidence on possible interference by well-known drugs and especially by those substances that are chemically related to the analyte, including possible metabolites of the analyte. The attention of kit users should be drawn to the publication of the American Association of Clinical Chemists on the effects of drugs on analytical assays (5).

5. **EFFECTIVENESS OF A KIT IN CLINICAL DIAGNOSIS**

If a diagnostic kit is developed and supplied for a specified clinical objective, then the effectiveness of the kit in achieving this objective shall be part of the assessment. Clearly this effectiveness will depend in large measure on the qualities indicated in sections 2–4 above.

In certain cases the effectiveness of a kit that gives a “Yes/No” result (e.g., one testing for pregnancy or for hepatitis antigen) can be
assessed by a relatively simple clinical trial and the data presented in
the form of rates of false-negative and false-positive results.

In the case of kits intended for a stated clinical purpose, the ex-
pected analyte concentration ranges in the defined population (refer-
ence range) and in defined clinical conditions should, where feasible,
be given. It is important for control authorities in different countries
to appreciate the extent to which analyte concentration ranges in
defined populations may differ from country to country (because of
genetic, dietary, environmental, and other differences) and within
population groups within a country (because of age, sex, occupational,
and other differences). It is the responsibility of the user to confirm
or establish normal ranges for the specific population in which the
test is being used.

The clinical effectiveness of a kit may also depend on other factors
unrelated to the quality of the kit per se. The manufacturer should
assess and provide information on the effect of these factors, which
include:

— possible differences between estimates in plasma and serum, and
  the effect of anticoagulants;
— conditions under which the specimen should be collected (e.g.,
  fasting, time of day, posture), separated (e.g., rapidly, cold), and
  stored (e.g., frozen at –20°C or below); and
— effects of interfering drugs and other substances (e.g., steroid-
  binding globulins in pregnancy) on the assay system itself and on
  the metabolism of the analyte.

6. CLINICAL TESTING OF ASSAY SYSTEMS

6.1 Assay systems previously well described

In the clinical testing of assay systems previously well described and
of endogenous substances on whose physiology information is available,
the expected range of normal values in a specified suitable population
shall be stated, including ranges obtained for stimulation (provoca-
tion) and suppression tests, if this is the proposed use of the kit. Such
analyte ranges shall be derived from assays on specimens taken from
adequate samples of well-specified populations and apparently normal
subjects, under carefully defined conditions. In addition, reference
shall be made to the influence of other stimuli that may be relevant.
For disease states the test kit is designed to study, adequate sampling is required, under defined conditions in a specified and preferably matched population, to permit reliable differentiation of pathological responses or values from normal ones. The distribution of values obtained in “normal” populations and the expected values in “diseased” populations shall be stated. Any overlap of the ranges in such populations shall be stated.

6.2 Assay systems for analytes previously undocumented

The clinical application of assay systems for analytes whose role in health and disease has not previously been documented requires separate special assessment. Results of assays carried out on specimens from groups of apparently normal subjects shall be provided as evidence in support of claims that the procedure might have a therapeutic or diagnostic application. Even when such applications are not known or claimed, evidence for the specificity of the assay system for the analyte shall be supplied. Any known limitation of the assay system or its application shall be stated. When a clinical application is claimed, the recommendations in section 3.4 shall be followed.

7. CLINICAL ASSESSMENT OF KITS FOR THE ASSAY OF DRUGS

In the assessment of a system for the assay of drugs, the drugs administered shall be of a quality approved by official agencies and published in established pharmacopoeias. International or national or, if these are not available, other well-recognized reference materials shall be used in the assessment of assays of drugs. The drug shall be administered in doses that provide adequate, but not excessive, therapeutic concentrations of the drug in plasma. The ability of the kit to assay the drug at plasma concentrations at which clinical decisions would be taken shall be shown by adequate sampling under well-defined conditions in specified populations. Knowledge of the pharmacodynamics and metabolism of the drug is helpful and desirable. Information provided shall include statements of the doses and dose forms of the drug, the time and route of its administration, and the times when test specimens for assay were obtained.
8. STABILITY OF KIT COMPONENTS AND KIT PERFORMANCE

The manufacturer shall assess—e.g., by accelerated degradation studies on reagents in their dry form and/or, after their reconstitution, as solutions kept under recommended storage conditions—the stability of (1) the individual components of the kit, before and after reconstitution (in the solution as directed), and (2) the assay performance characteristics of the whole kit when used according to the manufacturer’s instructions. Information about such testing, the number and results of the tests, and the statistical procedures used for their evaluation shall be made available to control authorities.

The performance characteristics for the shelf-life of the kit described above shall be provided. Kit batches incorporating different filling lots of components (e.g., analyte tracers) shall also be tested and the number of such tests, their results, and the statistical procedures used shall be stated.

Assay-to-assay reproducibility will be affected by the stability of the kit; in part, this will be a result of the changing bias or specificity of the kit as the least stable component deteriorates.

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ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice and for supplying additional data relevant to these requirements:

Dr E. H. Belcher, Medical Applications Section, Division of Life Sciences, International Atomic Energy Agency, Vienna, Austria

Dr R. C. Boguszinski, Director, Immunochemistry Laboratory, Miles Laboratories Inc., Elkhart, IA, USA

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REFERENCES


Appendix

GLOSSARY

Accelerated degradation studies

Studies in which an estimation of rate of change—for example, of a reagent—is made by comparing samples of the reagent subjected to conditions (e.g., high temperature) that increase the rate of change with samples kept under conditions at which such changes are minimal—e.g., at low temperature. (See Stability.)

Analyte

Substance in test specimen to be determined.

Analyte tracer

Analyte-incorporating tracer.

Analyte specificity

The specificity of an assay system for estimation of a given analyte in a given type of test specimen—e.g., a specified biological fluid.

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Assay kit

A set of components (reagents and other necessary materials) and procedural instructions packaged together and designed for the estimation *in vitro* of a specified analyte to a stated degree of precision, when used according to instructions.

Assay performance requirements

Assay performance characteristics required for a particular (specified) purpose.

Assay series, assay run, or assay batch

A set of estimates determined with the same reagents and reagent solutions under the same uniform conditions; thus, a group of assay tubes (of calibrator, test, blank, and control samples) that are handled together at the same time under the same conditions. The term “assay” is sometimes loosely used instead of “assay series”, “run”, or “batch” and this usage should be discouraged.

Assay standardization

Process of ensuring uniformity of assay results. By reducing bias between assay series or between laboratories, standardization helps to achieve better reproducibility of assay results and valid comparability. One measure usually taken (and often essential) for this purpose is the use of a calibration material (standard); in protein-binding assays (and many other assays involving biological systems) this may be insufficient by itself to ensure uniformity. In such instances, it may be necessary to define the assay reagents and procedure.

Assay system

All the components and procedures of an assay.

Assayist

Person responsible for the design and performance of an assay.
Batch

A group of specimens or set of containers processed together in a uniform way. The material in the containers may be similar, as in a uniform filling lot (as in pharmaceutical practice), or different, as in an assay series of various specimens.

Between-assay variation

See Reproducibility.

Bias

The numerical difference between the average of a series of estimates and the true or accepted value. This is equivalent to the definition of inaccuracy given by the International Federation of Clinical Chemistry.

Binding protein, binding reagent, binder

Protein used to quantify or detect an analyte by virtue of its ability to bind with it. Protein that reversibly and noncovalently binds a ligand and may be used to quantify or detect an analyte by virtue of its ability to bind with it. Examples include hormone cell receptor proteins, and plasma proteins that bind small molecules.

Biological reference material (or biological standard)

A reference material, generally of biological origin, which cannot be, or is not, characterized completely by chemical and physical means alone and which is needed for calibration or assessment of specificity.

The term “standard” is also often used to mean a set of specifications of the quality of reagents of an assay kit, or quality of assay performance.

Biological standard

See above.
Bound tracer

Total tracer present in the protein-bound fraction after separation. An uncorrected estimate normally includes the signal contributed by nonspecific binding and background.

Bound fraction

The portion of the incubation mixture which, after separation, contains the analyte bound to the binder protein.

Calibration curve

The relationship displayed graphically between the amount (dose meter) of reference material (horizontal axis) and the response of the detector (response meter) (vertical axis). (The term “standard curve” has been used, but should be discouraged wherever there could be ambiguity.)

The dose may be expressed as amount of specified substance or mass of the reference chemical, or as amount (“units”) defined by a specified material.

Dose meter is a transformation of the numerical value of the dose variable, usually log dose. The response meter is a transformation of the numerical value of the response variable, usually adopted either to linearize the log dose relation or to stabilize the variance.

The response variable can be, for example, the (uncorrected) activity signal in the free or bound fraction.

Calibration of kits

Steps taken (by manufacturer or laboratory) to ensure that the results of assays obtained with an assay kit are expressed in terms of mass of a pure chemical or, for substances that are incompletely defined, in terms of units defined by a reference material (q.v.).

Calibration material, calibrator

Material used for calibration in an assay series.
Carrier

1. Substance added to stabilize an assay reagent—e.g., by prevention of nonspecific absorption to surfaces—but which is otherwise inert towards the other reagents in the assay system. Analyte-free serum or plasma or enzyme-free albumin is generally used.

2. Support material for antigen or antibody in assay systems employing a solid phase.

3. In radiochemistry, nonradioactive nuclide present or added to dilute radionuclide—e.g., to minimize absorption or biological uptake.

Clinical effectiveness

Extent to which an assay fulfils the clinical purpose for which it was carried out.

Consensus value

Agreed value (e.g., for concentration or activity) derived from many, preferably independent, assays or observations.

Cross-reaction

Ability of substances other than the analyte to bind to the binding reagent, and ability of substances other than the binding reagent to bind to the analyte.

Such substances, if present in a test sample, may compete with the analyte for the binding site—thus leading to an erroneous potency estimate.

These substances may be natural precursor forms of the analyte or binding protein, degradation products (from in vivo or in vitro degradation), or other substances that carry on their surface a molecular configuration similar to the binding (immunoreactive) site(s) of the analyte or binding protein.

Detectability, detection limit

The smallest amount or concentration of analyte which, with stated confidence (commonly two standard deviations, or expressed as confidence or fiducial limits), can be distinguished from zero. This
value depends upon the precision of the measurements of zero dose solution and of the specimen. (See Sensitivity.)

In some detector systems the detection limit is determined by the signal-to-noise ratio in the measurement device.

**Dose-response curve**

See Calibration curve.

**Filling lot**

Group of containers filled with reagent of the same batch by the same filling equipment under uniform conditions in one continuous operation.

**Free fraction**

The portion of the incubation mixture which does not contain the bound analyte complex. (See Bound fraction.)

**House standard**

Reference material (q.v.) generally prepared and used by a single manufacturer or laboratory.

**Immunoassay**

Term used for an assay procedure based on the reversible and noncovalent binding of an antigen by antibody. Immunoassays can be employed to detect or quantify either antigens or antibodies.

**Immunogen**

Substance that, when administered under appropriate conditions to a suitable animal, will stimulate the production of antibody, or antibodies, that can combine reversibly and noncovalently with the immunogen as antigen.

All immunogens are antigens; however, not all haptens are immunogenic and able to stimulate a specific antibody response.
Immunoreactivity

A term commonly used for the ability of a specified antigen to combine with an antibody, or a specified antibody to combine with an antigen.

The term "immunological activity" is loosely used to denote: (1) the ability of an immunogen to elicit an immune response (e.g., to raise antibodies); (2) the "potency" of an antigen exhibited in an immunoassay; or (3) the potency of an antibody in an immunological reaction.

Interference

Effect of factors or substances (other than the cross-reactants) that bias assay results, either by affecting the kinetics of the reaction or by altering the efficiency of the separation procedure.

The term "nonspecific interference" has also been used.

Should be distinguished from "cross-reaction" (q.v.) which refers only to substances (other than the analyte, labelled antigen, and antibody) interfering with the binding reaction due to steric similarity.

International reference material

Reference material (q.v.) distributed on an international basis by a recognized international or, in some cases, national organization.

Kit

See Assay kit.

Kit component

Material included in an assay kit. Primary components of a typical kit are: (1) the binding reagent (q.v.); (2) the tracer (q.v.); and (3) the calibrator; secondary components include the separation materials, buffers, and quality control sera (if included), hardware, and instructions.

Label

1. Paper bearing information attached to the immediate container of a reagent.

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2. The substance (e.g., radionuclide, fluorophor) attached to one of the assay reactants for the purpose of facilitating observation of the binding reaction by yielding a perceptible signal.

**Labelled binding protein**
Tracer (q.v.) in which the labelled reactant is the binding protein.

**Labelled ligand**
Tracer (q.v.) in which the labelled reactant is the ligand.

**Ligand**
Substance that is reversibly and noncovalently bound by a binding agent. A general term used for analyte, cross-reactant, or calibrant that binds to the binding reagents.

**Mass concentration**
Mass of a component (e.g., solute) divided by volume of system (e.g., solution).

**Maximum binding**
Term used in two senses:
1. The amount (usually expressed as a percentage) of labelled ligand bound to excess binding reagent.
2. The maximum amount (usually expressed as a percentage) of labelled ligand bound by the binding reagent at its working dilution in the absence of added calibrant or analyte (zero dose binding).

**Monoclonal antibodies**
Antibodies derived from a single clone of lymphocytes.

**Nonspecific binding**
The fraction of labelled material present in the bound fraction for reasons other than specific binding to the binding site of a binding protein.
Operator

Person carrying out an assay (cf. Assayist).

Parallelism

Extent to which dose-response curves (q.v.) of two substances are identical, except for displacement along dose axis of one relative to another. If the curves are curvilinear, this condition is described as "generalized parallelism". Parallelism is one test of identity of two preparations—e.g., of analyte and calibrator. It is a prerequisite for the calculation of a single valid value of relative potency of one substance compared with another in an assay. Strictly, it applies only when the dose-response curves are linear.

Performance characteristics (of an assay)

Properties of an assay system relating to reliability and practicability. Characteristics of the reliability of an assay include precision, bias, sensitivity, specificity, validity, and ruggedness.

Characteristics of the practicability of an assay include speed, technical simplicity, cost, resources required, service availability.

Population range (of analyte concentration)

The range of estimated concentrations of an analyte (or presumed analyte) in a stated biological fluid (assayed in a particular manner) that is found in a defined population. The population may be defined with regard to relevant factors such as age, sex, geography, ethnic group, diet, drug treatment, social levels, physiological status, and the time, manner, and conditions in which the specimen of biological fluid is withdrawn.

Precision/Imprecision (of a measurement)

Precision is the closeness of agreement between the results obtained by applying a given experimental procedure several times under prescribed conditions.

As precision has no numerical value, the term imprecision, to which a numerical value can be assigned, may be preferable in some contexts.
Imprecision consists of disagreement between, or variability among, replicate measurements (of the same material), and is usually expressed as the standard deviation or variance or coefficient of variation. Such variability can occur within a single assay series, between assay series, between batches of reagents, between operators or laboratories, and between assay systems. Confidence limits or fiducial limits quantify the uncertainty about the value of a parameter after estimation from an experiment or sample.

Whenever a figure for variability is given, it should be stated whether it applies within a single assay series, between assay series, between batches of reagents, between operators or laboratories, or between assay systems.

In protein-binding assays, the variability of the estimate(s) may depend on the analyte concentration(s) measured. (See Precision profile.)

**Precision profile**

Graphical representation of precision (or imprecision) of measurements obtained with an assay system over a range of dose levels of the analyte. Such profiles are of value in assessing the reliance that can be placed on an assay estimate at a particular dose level, because the precision in binding assays may vary considerably with analyte concentration.

In assays where the coefficient of variation in dose measurement is constant over the dose range used, the performance of the assay system may be represented by a single index of precision.

**Quality assessment**

Measures taken to evaluate the quality of reagents, kits, assay systems, and laboratories.

**Quality assurance**

The use of quality control and explanatory measures to ensure both the maintenance of quality of reagents, kits, and assay systems and the availability of appropriate performance data to users, peer laboratories, and others.
Quality control

The use of appropriate measures to detect, trace, and eliminate (or reduce to an acceptable level) all systematic variations in the quality of reagents, kits, and assay systems, and thus maintain or improve their quality.

Quality control, internal or within-laboratory

Measures taken within a laboratory to ensure that the assay system gives acceptable and reproducible results. These normally involve the measurement of precision and bias within an assay series, or between assay series.

Quality control, external or between-laboratory

External quality control comprises those statistical and supportive measures taken by an independent organization both to assess the performance of individual laboratories and to reduce interlaboratory bias and improve within-laboratory precision.

Quality control material

Specimens of body fluids—e.g., serum, plasma, and urine—used to assess and control the quality of (assay) reagents or assay performance. Samples from pools of such material are included in assay runs to assess between-assay and between-laboratory reproducibility. The material is also used by assay kit manufacturers for the quality assessment of assay reagents and assay systems.

Recovery

The amount of analyte measured in the assay system expressed as a percentage of the amount of analyte originally present in the specimen. Usually estimated by addition of a known amount of radioactive or inactive analyte.

Reference material

A material or substance, one or more properties of which are sufficiently well established to be used for the calibration of an apparatus,
for the assessment of a measurement method, or for assigning values to materials. Reference materials may be distributed on a laboratory, regional, national, or international basis.

Reference preparation

Identified preparation of a reference material of attested suitability containing a specified analyte and intended for assessment of quality or quantitation of an assay system.

Essential prerequisites for a reference preparation are that each sample of it is identical to another, and that its stability should be measured and suitable for its intended use.

Reference range

A range of concentrations that includes a stated percentage (usually 95% or 99%) of a defined population group. The range may be one-sided (e.g., when only high values are considered abnormal), or two-sided (excluding, say, the lowest $2^{1/2}\%$ and the highest $2^{1/2}\%$ of the population when both high and low concentrations are of clinical interest).

The reference range is most satisfactorily estimated from the percentiles of the set of concentrations obtained for a group of individuals, chosen to represent the defined population. This takes into account any skewness of the distribution of values. The precision with which the reference range is estimated will depend on the number of individuals in the group—and confidence limits can be assigned to the boundaries of the range. The range may be biased if the group of individuals is not a random sample of the defined population—i.e., if not all members of the population have an equal chance of being included.

Reference serum

Serum containing one or more specified analytes accepted as suitable for assays for the comparison and assessment of bias and reproducibility of different assay systems. Such a serum may be distributed on a laboratory, regional, national, or international basis.
Reproducibility

Closeness of agreement between the results of measurements of the same quantity, where the individual measurements are made: by different methods, with different measuring instruments, by different observers, in different laboratories, after intervals of time quite long compared with the duration of a single measurement, under different normal conditions of use of the instruments employed.

Can be expressed quantitatively as standard deviation or coefficient of variation and may be referred to as between-assay series variation. Reproducibility may be dependent on dose and thus may vary at different analyte concentrations.

Robustness (of data-processing procedures)

Insensitivity of estimates and statements of precision to small variations in assumptions used in statistical analysis such as choice of metameters and form of dose-response relation.

Ruggedness

Characteristic of an assay system that makes the results obtained unaffected by changes in assay reagents and procedures.

Ruggedness can be assessed experimentally by observation of the influence on assay results (either from assay tube to assay tube or from assay batch to assay batch) or changes in the amount or quality of the assay reagents, or in the assay procedure.

In practice, nonruggedness is manifested by poor precision, poor interassay variability, and poor interlaboratory agreement.

Separation material

Reagents by which the labelled tracer fraction is separated from the bound fraction in a binding assay system.

Separation procedure

Procedure by which the free fraction is separated from the bound fraction in a protein-binding assay.
Separation system

Reagents and procedure used to separate bound and free fractions.

Shelf-life (of batch of kits)

Period from manufacture until recommended expiry date of the batch of kits.

Specificity

The specificity (structural) of a protein-binding reagent is the degree to which it is not influenced by cross-reacting substances.

The specificity of an assay system is the degree to which the results are not influenced by cross-reacting substances, or other noncompeting substances such as plasma proteins and small ions present in the system, or other factors, such as the pH or the temperature of incubation, that affect the reaction.

Specificity may be evaluated: (a) by the introduction of potential cross-reacting substances and other substances likely to affect the reaction; and (b) by examination of the similarity of the performance of the assay system with test specimens and with reference materials, under minor differences of assay conditions—e.g., at different concentrations of analyte or parallelisms.

Specific activity

Activity per unit mass of substance—e.g., μCi/mg; Bq/mg.

Stability

Lack of alteration under defined conditions. Thermal stability of, for example, a reagent may be quantified as rate of chemical change at stated temperature(s). Often measured by accelerated degradation studies involving comparisons of samples of a material maintained at different temperatures for one or more periods of time.

Substance concentration

Amount of substance of a solute divided by volume of solution (expressed as mol/l).
Test specimen

Specimen (e.g., of biological fluid) in which it is required to detect or quantify the analyte.

Titre

Term for the volume fraction (or dilution) of a specimen or other solution at which a specified effect or end-point is observed. Usually expressed numerically as the reciprocal of the dilution.

In the context of an immunoassay, titre is usually used in the assessment of antiserum for the final dilution at which a specified substance fraction (percentage) or a specified amount of a specified analyte is bound.

The numerical value of a titre of, for example, an antiserum is thus influenced by the amount and affinity of antibodies and the conditions under which the test is made.

Tracer

The tracer is the reactant (ligand or binding protein) incorporating a label which is used to trace the distribution of the unlabelled reactant in an assay system.

Within-assay series, run, or batch precision

Precision determined in an assay series, run, or batch.

Within-assay series, run, or batch sensitivity

The detection limit determined in an assay series, run, or batch.

Working range of assay

Range of analyte concentration in specimens for which the assay system gives results with precision acceptable for the purpose (e.g., clinical) for which the assay was carried out.
Annex 9

GUIDELINES FOR QUALITY ASSESSMENT
OF ANTITUMOUR ANTIBIOTICS

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INTRODUCTION

1. Historical

In 1963 the seventeenth WHO Expert Committee on Biological Standardization was informed that there might be a need for international reference preparations of the actinomycins (1, p. 11). The Committee requested the WHO Secretariat to obtain more information on these substances. It was found that actinomycins C and D and mitomycin C were of limited clinical interest as chemotherapeutic agents for cancer (2, p. 9). Subsequently, the possibility that international reference preparations of other antitumour antibiotics might be needed was raised with the nineteenth, twenty-first, and twenty-third WHO Expert Committees on Biological Standardization (olivomycin A (3, p. 12),mithramycin (4, p. 15), daunorubicin (4, p. 15), bleomycin (5, p. 13)). During this period, there was interest in whether any of these substances could be characterized completely by chemical and physical means, since this had a bearing on whether biologically standardized reference preparations would be required.

In its continuing effort to collect comprehensive information on antitumour antibiotics, the WHO Secretariat in 1974 circulated a questionnaire to experts in 22 countries concerning the medical use of antitumour antibiotics and requesting opinions concerning the necessity for establishing international reference preparations of them.

Replies from experts in 18 countries indicated that antitumour antibiotics were used in their respective countries. As shown in Table 1, it was reported that bleomycin was used in 15 countries, daunorubicin in 14, dactinomycin in 13, doxorubicin in 9, mitomycin in 7, mithramycin in 6, chromomycin in 4, rufocromomycin in 3, and carubicin, carzinophilin, and sarcomycin each in only one country.

Replies from 20 countries expressed a need for international biological reference preparations of antitumour antibiotics. A number of comments stated a preference for activity to be expressed in terms of biological units.

The twenty-seventh WHO Expert Committee on Biological Standardization recognized that the need for international reference materials to be used in the control of antitumour antibiotics was becoming more urgent (6, p. 10). Assays for antibacterial activity were part of national specifications for certain antitumour antibiotics, and international specifications for bleomycin, doxorubicin, and other antitumour antibiotics were being prepared (6, p. 10; 7, p. 16).
### Table 1. Antitumour antibiotics used in various countries (1974)

<table>
<thead>
<tr>
<th>Country</th>
<th>Bleomycin</th>
<th>Carboplatin</th>
<th>Carzinophilin</th>
<th>Chromomycin</th>
<th>Dactinomyycin</th>
<th>Daunorubicin</th>
<th>Doxorubicin</th>
<th>Mitomycin</th>
<th>Mitomycin</th>
<th>Rufomycycin</th>
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*One other country reported that almost all the antitumour antibiotics are used, but especially dactinomycin.
2. Recent Growth of Chemotherapeutic Use of Antitumour Antibiotics

There is a growing list of antitumour antibiotics that are at present effectively used in cancer chemotherapy. Chemotherapy is of increasing importance in cancer management, and information on products used for cancer chemotherapy should be readily available to doctors in all countries. In treating the early stages of cancer, chemotherapy in conjunction with surgery and/or radiotherapy frequently prevents the occurrence of metastases. The subject is of particular significance for developing countries because, in the advanced stages of cancer, chemotherapy is the main weapon and it requires less technical equipment and manpower than do surgery and radiotherapy.

Antitumour antibiotics are at present widely used in cancer chemotherapy. Doxorubicin is one of the newest agents discovered to have activity in breast cancer and it has one of the highest reported response rates among single agents—more than 40% in patients not previously treated with chemotherapy (8, p. 29). It is one of the best and most easily administered single agents for the treatment of metastases in osteogenic sarcoma, and produces a 30% response rate when used alone. Carubicin is effective in the treatment of soft tissue sarcomas and in breast cancer.

Bleomycin has shown activity against squamous cell carcinoma of the head and neck. Squamous cell carcinoma of the epidermis is the most common cancer of the skin and the most common cancer in man. Bleomycin is effective for systemic treatment and has been used extensively.

Daunorubicin and carubicin are used in the treatment of acute leukaemia, as well as in malignant reticulosities and uterine chorioepithelioma. Rufocromomycin is used in the treatment of Hodgkin’s disease, reticulocytosis, and kidney adenocarcinoma, as well as neuroblastoma (sympathogonioma) resistant to other cytostatics. Olivomycin and mithramycin have been recommended for use in the following diseases:

(1) Testicle tumours (seminomas, embryonal cancers, teratoblastomas) at the stage of dissemination (metastases to the lungs, the abdominal organs, and the lymphatic nodes).
(2) Tonsillar tumours.
(3) Reticulosarcomas with involvement of peripheral nodes.

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Dactinomycin is used in Stages I and II of nephroblastoma (Wilms' tumour), the second most common abdominal malignant solid tumour in children.

REFERENCES


QUALITY REQUIREMENTS

1. International Specifications

International specifications for quality control of pharmaceutical preparations have traditionally been formulated for materials in wide clinical use, usually at a time when national specifications have been published in several countries. The international specifications have then been based on a synthesis of the most useful information from official national sources. In the case of antitumour antibiotics, however, it has not been possible to follow this course of action. Although many of the materials have been in use for more than 10 years, this use has been very limited, often to such an extent that the materials have not been available commercially, their issue being restricted by state authorities and the manufacturers.

The survey made by WHO at the request of the Expert Committee on Biological Standardization (WHO Technical Report Series, No. 463, 1971, p. 13) showed that only two countries had published requirements or specifications for any of the antitumour antibiotics—namely, Japan and the USA. These specifications were primarily concerned with biological quality and were based on a knowledge of manufacturing processes, obtained by inspection, which is not indicated in the published requirements. None of the requirements described a biological assay of antitumour activity. It was thought that the need for an assay of antitumour activity could be avoided if a sufficiently detailed chemi-
cal and physical specification could be made, in the first instance for bleomycin and doxorubicin, and that the provision of such specifications might be undertaken by WHO. These two materials were chosen to represent respectively the problems of antitumour antibiotics consisting of a complex mixture of active components and those that are highly purified, homogeneous, and crystalline; the provision of monographs for these materials is being undertaken. A number of important questions relating to the supply of international reference materials will need to be answered before these international specifications can be completed. It is unlikely that similar international specifications can be drawn up in the foreseeable future for the nine other antitumour antibiotics that are in international use.

It is hoped that the provision of “Guidelines for quality assessment of antitumour antibiotics” will assist national authorities in assuring the quality of antitumour antibiotics until definitive international specifications can be agreed.

2. Guidelines

Information and specifications are provided in these Guidelines in relation to bleomycin, bleomycin A₅, carubicin, chromomycin A₃, dactinomycin, daunorubicin, doxorubicin, mitramycin, mitomycin, olivomycin, and rufocromomycin. These substances were chosen as those most commonly used. In many cases only a single source of manufacture exists, but in all cases control of quality is exercised by at least one national authority on the basis of minimum specifications, which are published or were made available to WHO. The data specified for the individual antibiotics are taken from these sources and additional information collected from published work has been added. When available, the reference has been given, but some data are taken from the files of the WHO Secretariat. When a monograph is prepared and published as an “International specification for pharmaceutical substances” (International Pharmacopoeia), this implies that all the data and test procedures have been examined and confirmed, independently, in several laboratories. The data and information provided in this annex have not been checked in this way so that they should be used with caution. The Guidelines, however, were circulated for comment to various experts. Where differences in relation to specific details of methodology or specifications exist, they are included in the text without comment.
3. Use of Guidelines

It is hoped that the information provided here will be of use to national control authorities who need to examine, or control, imported antitumour antibiotics and their preparations. It was not the intention to provide full experimental details, as in pharmacopoeias, but to draw attention to aspects of the quality control that are considered to be important and for which detailed information is available elsewhere. Further information to allow certain tests to be carried out may need to be obtained from the references given, or from the manufacturer. If difficulty is experienced in obtaining such information, the WHO Secretariat may be able to assist. Many of the tests require the use of an authentic sample or of a biological reference preparation with a defined activity. Except for bleomycin, these are not available as WHO international preparations but a list of national reference preparations that have been established is included.

If the national control authority of the importing country is not able to perform all the tests described, the Guidelines indicate those aspects of quality control for which authenticated experimental data should be available in the manufacturer's records.

4. Caution in Handling Materials

Antitumour antibiotics are very toxic and should be handled with great care. All dry powders should be transferred in a suitable protective cabinet. Laboratory personnel should wear rubber gloves and avoid inhaling fine particles of powder. Solutions should not be pipetted by mouth. If the substance comes in contact with the skin, it should be washed with soap and water. All waste material should be disposed of by treatment with hypochlorite solution and dilution with large volumes of detergent solution.

5. Specifications for Antitumour Antibiotics

BLEOMYCIN

Synonym: Bleomycin complex A2/B2
Structure (see overleaf):
Bleomycin A₂:  \( R = \text{NH} - \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{S} - \text{CH}_3 \)

Bleomycin B₂:  \( R = \text{NH} - \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{NH} - \text{C} - \text{NH}_2 \)
Produced by *Streptomyces verticillus* and available as bleomycin hydrochloride or sulfate.

The following tests and specifications are equally applicable to both forms unless otherwise noted.

**IDENTIFICATION TESTS**

1. Ultraviolet absorption. To 4 mg of bleomycin hydrochloride add 5 µl of copper sulfate test solution and enough water to effect solution; make up to 100 ml. The solution shows the absorption maxima at 240–243 nm and 288–293 nm, and the minimum at 267–270 nm when determined in a 10-mm cell.

2. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of bleomycin per 200 mg of KBr, the spectrum obtained resembles that of an authentic substance, similarly treated. The infrared spectrum (see Appendix) of an authentic sample of bleomycin sulfate may be used for comparison.

3. Sakaguchi test: positive (3).

**PURITY TESTS**

1. pH: a 5 mg/ml solution in water has a pH of 4.5–6.0 (1, 2).

2. Loss on drying not more than 6.0% (1) or 3.0% (2) (bleomycin sulfate) or 5.0% (2) (bleomycin hydrochloride).

3. Limit of copper: not more than 0.1% (1, 2).

4. Pyrogens, vasodepressor substances and abnormal toxicity (see section 7, "Problems in quality assessment of antitumour antibiotics").

5. Content of bleomycin components. Using column chromatographic separation by a gradient elution technique (1, 2, 4) followed by measurement of the ultraviolet absorption of separated components, the following limits are required, expressed as percentages of total bleomycin components.

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<tr>
<th>Component</th>
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<td>B₂₉</td>
<td>25–32% (1, 2)</td>
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<td>Bₙₙ</td>
<td>not more than 1% (2)</td>
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<td>not more than 35% (2)</td>
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<td>A₂ + B₂</td>
<td>not less than 90% (1)</td>
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**BIOLOGICAL ASSAY**

Potency: The biological activity may be defined in weight equivalents as in Japan (2):

"the bleomycin standard contains 1 mg of bleomycin (potency) per 1.03 mg of the Japanese bleomycin master standard" (which is Bleomycin A₂.

Or in units as in the United States of America (5):

"one unit of bleomycin activity (potency) is contained in 0.637 mg of the United States of America bleomycin master standard" (which is Bleomycin A₂).

In the United States of America, the official working standard is a preparation of bleomycin complex A₂/B₂.
There is an international reference preparation for bleomycin.

The potency may be measured by agar diffusion assay, using a suitable test organism, e.g., Bacillus subtilis (ATCC 6633) or Mycobacterium smegmatis (ATCC 607) (1, 2).

The potency should lie between 1400 and 2000 μg/mg (2) or between 1.5 and 2.0 units/mg (1). (μg” are microgram equivalents based on activity.)

**DOSEAGE FORM**

Bleomycin is available in an injectable dosage form.

It is not usual for bleomycin to have a carrier in the dosage form.

The tests applied to the antibiotic that are applicable also to the dosage form are the following:

1. Ultraviolet absorption.
2. pH: a 5 mg/ml solution in water has a pH of 4.5–6.0 (1) or 4.5–6.5 (2).
3. Loss on drying: not greater than 9% (2) (bleomycin hydrochloride) or 6% (J, 2) (bleomycin sulfate).
4. Limit of copper.
5. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, “Problems of quality assessment of antitumour antibiotics”).
6. Composition.
7. Potency.

**ADDITIONAL TESTS FOR THE DOSAGE FORM**

1. Biological assay: the vial contains 90–125% (2) of the labelled potency, or 90–120% (J) of the labelled potency.
2. Sterility: test by a membrane filtration technique (6).

**REFERENCES**

1. United States Code of Federal Regulations, Title 21, Section 450.10a.
5. United States Code of Federal Regulations, Title 21, Section 430.6 (a) (5).

**BLEOMYCIN A<sub>5</sub>**

Synonym: Bleomycetin

Structure (see facing page):

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\[ R = \text{NH} - \text{CH}_2 - \text{CH}_2 - \text{NH} - \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{NH}_2 \]
Produced by *Streptovorticillium griseocarneum* var. *bleomycini*.

IDENTIFICATION TESTS

1. Ultraviolet absorption. A 0.10 mg/ml solution in water exhibits maxima at about 244 nm and 292 nm and a minimum at about 269 nm (1).
2. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of bleomycin A₅ per 200 mg of KBr, the spectrum obtained resembles that of an authentic substance, similarly treated (2). The infrared spectrum (see Appendix) of an authentic sample of bleomycin A₅ sulfate may be used for comparison.
3. Sakaguchi test: negative (3).

PURITY TESTS

1. pH: a 5.0 mg/ml solution in water has a pH of 5.0–6.5 (1).
2. Water (Karl Fischer): not more than 6.0% (1).
3. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, “Problems in quality assessment of antitumour antibiotics”).
4. Content of bleomycin A₅. Determine using column chromatographic separation (4) by a gradient elution technique followed by the measurement of ultraviolet absorption of separated components.
   Bleomycin A₅, not less than 85% (1), expressed as a percentage of total bleomycin components.
5. Chemical assay. Determine the absorbance of a 0.10 mg/ml solution in water in a 1-cm layer at the maximum at about 292 nm and calculate the content using an absorbivity value (1 g/litre, 1 cm) of 14.3. It contains not less than 85% on the basis of the anhydrous substance.

BIOLOGICAL ASSAY

Potency: the biological activity may be defined in weight of the pure material. The bleomycin A₅ reference substance contains 100% of bleomycin A₅. There is no international standard or chemical reference substance for bleomycin A₅.

The potency may be measured by agar diffusion assay using a suitable test organism, e.g., *Bacillus subtilis* (ATCC 6633).

The potency of bleomycin A₅ is not less than 85% of the reference substance on an anhydrous basis (1).

DOSAGE FORM

Bleomycin A₅ is available in an injectable form. It is usual for the dosage form to contain mannitol as a carrier. The tests applied to bleomycin A₅ that are also applicable to the dosage form are the following:
1. Ultraviolet absorption.
2. pH.

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3. Water (Karl Fischer): the content shall be not greater than 6% (I).
4. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, "Problems in quality assessment of antitumour antibiotics").
5. Content of bleomycin A₂.
6. Chemical assay (for vial content): contains 90–125% of the labelled content (I).

ADDITIONAL TESTS FOR THE DOSAGE FORM

1. Biological assay: the vial contains 90–125% of the labelled potency (I).
2. Sterility: test by a membrane filtration technique (J).

REFERENCES

1. Instructions for control, prepared by the Institute of New Antibiotics of the Academy of Medical Sciences of the USSR.
2. United States Code of Federal Regulations, Title 21, Section 450.10a.

CARUBICIN

Synonym: Carminomycin

Structure:

![Structure of Carubicin]

Produced by: Actinomadura carminata and available as carubicin hydrochloride.
IDENTIFICATION TESTS

1. Physical appearance: a red-orange powder.
2. Ultraviolet absorption. A 0.02 mg/ml solution in methanol exhibits maxima at about 236 nm, 255 nm, 462 nm, 478 nm, 492 nm, 510 nm, and 525 nm with an absorbivity value (1 g/litre, 1 cm) of 30.0 at the maximum at 492 nm.
3. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of carubicin per 200 mg of KBr, a spectrum is obtained resembling that of an authentic substance similarly treated. The infrared spectrum (see Appendix) of an authentic sample of carubicin may be used for comparison.
4. Specific rotation: \([\alpha]_D^{20} = +289^\circ (c = 0.18 \text{ in methanol}) (1)\).
6. Chromatography. The material behaves in a manner similar to that of an authentic substance, using the following conditions:
   - (a) silica gel as coating substance and development with methylene chloride:methanol:water in the proportions of 100:20:2; doxorubicin, daunorubicin, and carubicin separate;
   - (b) using silica gel as coating substance and development with chloroform:methanol:glacial acetic acid in the proportions 80:20:5.
7. Colour test. To 2 mg in 2 ml of methanol add 2 ml of water and one drop of sodium hydroxide solution (80 g/litre); orange-red solution turns blue-violet.

PURITY TESTS

1. pH: a 5.0 mg/ml solution in water has a pH of 3.8–6.5.
2. Water (Karl Fischer): not more than 4.0% (4).
3. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, “Problems in quality assessment of antitumour antibiotics”).
4. Limit of content of related substances. No individual related substance greater than 20 μg/mg estimated as carubicin when determined by thin-layer chromatography on 100 μg as described in 6 (b) above.
5. Chemical assay. Determine the absorbance of a 20 μg/ml solution in methanol in a 1-cm layer at the maximum of about 49 nm and calculate the content using an absorbivity value of 30.0; the content is not less than 90% on the basis of the anhydrous substance.

BIOLOGICAL ASSAY

Potency: the biological activity may be defined in weight equivalents.

The potency may be measured by agar diffusion assay using a suitable test organism, e.g., *Bacillus subtilis* (ATCC 6633). The potency is not less than 900 microgram equivalents/mg and not greater than 1100 microgram equivalents/mg of carubicin hydrochloride, on an anhydrous basis.

There is no international standard or chemical reference substance for carubicin.

DOSE FORM

Carubicin hydrochloride is available in an injectable form. It is usual for the dosage form to contain mannitol as an inert carrier.

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The tests applied to the antibiotic that are also applicable to the dosage form are the following:
1. Physical appearance.
2. Ultraviolet absorption.
3. pH.
5. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, "Problems in quality assessment of antitumour antibiotics").

ADDITIONAL TESTS FOR THE DOSAGE FORM

2. Biological assay: the vial contains 90–115% of the labelled potency.

REFERENCES

4. Instructions for control, prepared by the Institute of New Antibiotics of the Academy of Medical Sciences of the USSR.
Synonym: Toyomycin

Structure:

Produced by: *Streptomyces griseus.*

**IDENTIFICATION TESTS**

1. Physical appearance: yellow powder.
2. Ultraviolet absorption. A 0.01 mg/ml solution in water exhibits maxima at about 230 nm, 280 nm, 302 nm, 318 nm, 330 nm, and 412 nm with absorptivity values (1 g/litre, 1 cm) of 20.8, 44.0, 6.0, 7.0, 5.8, and 10.0 at the respective maxima (7). In ethanol, maxima are exhibited at about 229 nm, 280 nm, and 318 nm (2).
3. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of chromomycin A₃ per 200 mg of KBr, a spectrum is obtained resembling that of an authentic substance similarly treated. The infrared spectrum (5) (see Appendix) of an authentic sample of chromomycin A₃ may be used for comparison.
4. Specific rotation: 
   - $[\alpha]_D^{20} = -55^\circ$ ($c = 1.0$ in ethanol) (4)
   - $[\alpha]_D^{20} = -45^\circ$ to $-60^\circ$ ($c = 1.0$ in ethanol) (2).
5. Melting point: 183 °C (2).
6. Chromatography. Thin-layer chromatography, 50 μg. The material behaves in a manner similar to that of an authentic preparation, using the following conditions:
   - Silica gel as the coating substance, developing with a mixture of chloroform:methyl ethyl ketone:ethanol in the proportions of 6:1:4, and detecting with ultraviolet light at 356 nm ($R_f$ about 0.3).
PURITY TESTS

1. Loss on drying: not more than 4.0% (2).
2. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, "Problems in quality assessment of antitumour antibiotics").
3. Chemical assay. Determine the absorbance of 0.01 mg/ml in water in a 1-cm layer at the maximum of about 280 nm and calculate the content using an absorptivity value (1 g/litre, 1 cm) of 44.0. It contains not less than 85%.

BIOLOGICAL ASSAY

Potency: the biological activity may be defined in weight of microgram equivalents of chromomycin A₃ (2).

The potency may be measured by agar diffusion assay using a suitable test organism, e.g., Bacillus subtilis (ATCC 6633) (2). The potency is not less than 850 microgram equivalents per mg (2). There is no international standard or chemical reference substance for chromomycin A₃.

DOSAGE FORM

Chromomycin A₃ is available as an injectable dosage form.

It is not usual for the dosage form to contain a carrier. The tests applied to the antibiotic that are also applicable to the dosage form are the following:

1. Physical appearance.
2. Ultraviolet absorption.
3. Infrared absorption.
4. Specific rotation.
5. Melting point.
6. Chromatography.
7. Loss on drying: not more than 2.0% (2).
8. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, "Problems in quality assessment of antitumour antibiotics").

ADDITIONAL TESTS FOR DOSAGE FORM

1. Biological assay: the vial contains 90–125 % of the labelled content (2).
2. pH: a 50 μg/ml solution in water has a pH of 4.6–6.5 (2).

REFERENCES

1. Antitumour antibiotics and their clinical use, Medexport, USSR p. 49.

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DACTINOMYCIN

Synonym: Actinomycin D

Structure:

Produced by: *Streptomyces parvulus*.

**IDENTIFICATION TESTS**

1. Physical appearance: bright red powder.
2. Ultraviolet absorption. A 0.03 mg/ml solution in methanol exhibits maxima at 240 nm and 445 nm. Absorbance at 240 nm is 1.3 to 1.5 times its absorbance at 445 nm.
3. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of dactinomycin per 1200 mg of KBr, a spectrum is obtained resembling that of an authentic substance similarly treated. The infrared spectrum (see Appendix) of an authentic sample may be used for comparison.
4. Chromatography. When subjected to circular paper chromatography, the material behaves in a manner similar to that of an authentic substance, using the following conditions:
   - (a) 10% aqueous sodium o-cresotinate: butyl acetate: butyl ether in the proportions of 4:3:1 (v);
(b) 10% aqueous sodium o-cresotate : butanol : di-n-butyl ether in the proportions of 5:2:3 (7);
(c) 2% aqueous naphthalene-β-sulfonic acid : ethylacetate : di-n-butyl ether in the proportions of 2:1:1, with the paper impregnated with the lower phase (2);
(d) 10% aqueous sodium o-cresotate : tetrachloroethane : di-n-butyl ether in the proportions 2:1:3 (3).

When subjected to thin-layer chromatography, the material behaves in a manner similar to that of an authentic substance, using the following conditions: silica gel as the coating substance, 50 μg load and development with:
(a) a mixture of n-butanol : glacial acetic acid : water in the proportions of 6:2:2 (Rf about 0.71), or
(b) a mixture of n-butanol : isobutanol : ethanol : water in the proportions of 35:35:15:20:10 (Rf about 0.7), or
(c) a mixture of chloroform : methanol in the proportion of 1:1 (Rf about 0.59).
5. Specific rotation: [a]_D^20 = −262° (c = 0.25 in 95% ethanol) (4)
   [a]_D^20 = −315° (c = 0.25 in methanol) (5)
   [a]_D^20 = on the basis of the dried substance −292° to −317°
   (c = 0.1 in methanol) (3).
6. Melting point: 235.5–236.5 °C (4).
7. Colour reactions: about 1 mg of dactinomycin in paraformaldehyde/sulfuric acid gives a red-violet colour.

PURITY TESTS

1. pH: 5.5–7.0 (saturated solution) (3).
2. Loss on drying (3 hours at 60 °C in vacuum): not more than 15% (3, 6).
3. Pyrogens and abnormal toxicity (see section 7, "Problems in quality assessment of antimour antibiotics") (2).
4. Limit of content of related substances: no individual related substance found when determined by thin-layer chromatography of 100 μg as described in identification test (4).
5. Chemical assay:
   (a) determine the absorbance of a 0.03 mg/ml solution in methanol in a 1-cm layer at the maximum at about 445 nm and calculate the content using an absorptivity value (1 g/litre, 1 cm) of 19.6 (4);
   (b) determine the absorbance of a 0.02 mg/ml solution in methanol in a 1-cm layer at the maximum at about 445 nm: the absorptivity (1 g/litre, 1 cm) is 19.8–21.6 (3);
   (c) determine the absorbance of a 0.025 mg/ml solution in pH 7.0 phosphate buffer solution in a 1-cm layer at the maximum at about 437 nm using an absorptivity value of 24.0.
   It contains not less than 95% of dactinomycin on the basis of the dried substance.

BIOLOGICAL ASSAY

Potency: the biological activity may be defined in terms of the weight of the pure material. The dactinomycin reference substance contains 100% of dactinomycin (7).

There is no international standard or chemical reference substance for dactinomycin.
The potency may be measured by agar diffusion assay using a suitable test organism, e.g., *Bacillus subtilis* (ATCC 6633).

It contains not less than 95% (6) or 90% (3) of daclomycin on the basis of the dried substance.

**DOSAGE FORM**

Daclomycin is available in an injectable form.

It is usual for the dosage form to contain mannitol as a carrier. The tests applied to the antibiotic that are also applicable to the dosage form are the following:

1. Physical appearance.
2. Ultraviolet absorption.
3. Chromatography.
4. pH: the pH of the dosage form when reconstituted for use is 5.5–7.0 (3, 6).
5. Loss on drying: not more than 4% (3, 6).
6. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, "Problems in quality assessment of antitumour antibiotics").
7. Limit of content of related substances.
8. Chemical assay (for vial content): it contains 90–120% of the labelled content (1).

**ADDITIONAL TESTS FOR THE DOSAGE FORM**

1. Biological assay: the vial contains 90–120% of the labelled content (6).
2. Sterility: test by a membrane filtration technique (8).

**REFERENCES**

7. United States Code of Federal Regulations, Title 21, Section 430.6 (b) (32).
DAUNORUBICIN

Synonyms: Daunomycin and rubomycin

Structure:

![Chemical structure of daunorubicin]

Produced by: *Streptomyces peuceticus* and *Streptomyces coerulescens* and available as daunorubicin hydrochloride.

**IDENTIFICATION TESTS**

1. Physical appearance: a red-orange powder.
2. Ultraviolet absorption. A 0.02 mg/ml solution in methanol exhibits maxima at about 233 nm, 253 nm, 290 nm, 450 nm, 495 nm, and 530 nm and minima at about 245 nm, 280 nm, and 350 nm, with absorptivity values (1 g/litre, 1 cm) of 65.8, 44.0, 14.5, 22.5, 22.3, and 12.4 at the respective maxima.
3. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of daunorubicin per 200 mg of KBr, a spectrum is obtained resembling that of an authentic substance similarly treated. The infrared spectrum (see Appendix) of an authentic sample may be used for comparison.
4. Specific rotation: $[\alpha]_D^{20} = +250^\circ$ to $+275^\circ$ ($c = 0.15$ in methanol) ($I$) 
   $[\alpha]_p^{22} = +217^\circ$ to $+227^\circ$ ($c = 0.2$ in 0.01 N ethanolic HCl) ($2$).
5. Chromatography. When subjected to thin-layer chromatography, the material behaves in a manner similar to that of an authentic substance, using the following conditions:
   
   (a) silica gel as the coating substance and development with methylene chloride: methanol: water in the proportions of 100:20:2. Doxorubicin, daunorubicin, and carubicin separate;
   (b) silica gel as the coating substance and development with chloroform:methanol: glacial acetic acid in the proportions of 80:20:5.
6. Colour test. Dissolve 1–2 mg in 5 ml of 1 mol/litre sodium hydroxide; a bluish-purple colour is produced.

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PURITY TESTS.

1. pH: a 5.0 mg/ml solution in water has a pH of 4.5–6.5.
2. Water (Karl Fischer): not more than 3.0%.
3. Loss on drying: not more than 8.0% (I).
4. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, “Problems in quality assessment of antitumour antibiotics”).
5. Limit of content of related substances. No individual related substance should exceed 10 µg/mg estimated as daunorubicin when determined by thin-layer chromatography of 100 µg as described in Identification Tests, 5 (b) above.
6. Chemical assay. Determine the absorbance of a 20 µg/ml solution in methanol in a 1-cm layer at the maximum of about 495 nm and calculate the content using an absorptivity value of 22.3. It contains not less than 90% on the basis of the dried substance.

BIIOLOGICAL ASSAY

Potency: the biological activity may be defined in weight equivalents.
The potency may be measured by an agar diffusion assay using a suitable test organism, e.g., Bacillus subtilis (ATCC 6633). The potency is between 900 and 1100 microgram equivalents of daunorubicin hydrochloride per mg on the basis of the dried substance, or not less than 842 and not more than 1030 microgram equivalents of daunorubicin base per mg on the basis of the anhydrous substance. There is no international standard or chemical reference substance for daunorubicin.

DOSAGE FORM

Daunorubicin is available in an injectable form.
It is usual for the dosage form to contain an inert carrier such as lactose or mannitol.
The tests applied to the antibiotic that are also applicable to the dosage form are the following:
1. Physical appearance.
2. Ultraviolet absorption.
3. pH: a 5.0 mg/ml solution in water has a pH of 5.0–6.5 (I).
4. Water (Karl Fischer): not more than 3.0%.
5. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, “Problems in quality assessment of antitumour antibiotics”).

ADDITIONAL TESTS FOR THE DOSAGE FORM

1. Identity: the material behaves in manner similar to that of an authentic substance in a thin-layer chromatography test, using silica gel as the coating substance and developing with chloroform:methanol:water in the proportions of 14:6:1.
2. Biological assay: the vial contains 90–115% or 90–125% (I) of the labelled potency.

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REFERENCES


DOXORUBICIN

Synonym: Adriamycin
Structure:

Produced by: Streptomyces peuceticus var. caesius and available as doxorubicin hydrochloride.

IDENTIFICATION TESTS

1. Physical appearance: a red-orange powder.
2. Ultraviolet absorption. A 0.02 mg/ml solution in methanol exhibits maxima at about 233 nm, 253 nm, 290 nm, 477 nm, 495 nm, and 530 nm and minima at about 245 nm, 280 nm, and 350 nm with absorptivity values (1 g/litre, 1 cm) of 65.8, 44.0, 14.5, 22.5, 22.3, and 12.4 at the respective maxima (I).
3. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of doxorubicin per 200 mg of KBr, a spectrum is obtained resembling that of an authentic substance similarly treated. The infrared spectrum (see Appendix) of an authentic sample may be used for comparison.
4. Specific rotation: $[\alpha]_D^{20} = +248$ (c = 0.1 in methanol) (I).
5. Melting point: 205 °C with decomposition (I).
6. Chromatography: the material behaves in a manner similar to that of an authentic substance, using the following conditions:

(a) thin-layer chromatography, load 50 µg, and:
(i) a mixture of cellulose and polyamide (1:1) as the coating substance prepared with phosphate buffer pH 5.4 and development with a mixture of n-butanol:isopropanol:isopropyl ether:acetic acid:water in the proportions of 12:2:2:3:15 (upper layer) (2), or
(ii) silica gel as the coating substance and development with chloroform:methanol:glacial acetic acid in the proportions of 80:20:5, or
(iii) silica gel as the coating substance and development with methylene chloride:methanol:water in the proportions of 100:20:2; doxorubicin, daunorubicin, and carubicin separate (1);
(b) high-pressure liquid chromatography (HPLC), load 5 µg, using a 30-cm column having an inside diameter of 4.6 mm and packed with a suitable reverse phase such as Micro-Bondapack C18; solvent composed of water:acetonitrile in the proportion of 69:31; mobile phase composed of this solvent adjusted to pH 2 with phosphoric acid; internal standard of 2-naphthalene-sulfonic acid (2 mg/ml) in the solvent; and an ultraviolet detector at 254 nm (3).

7. Colour test: to 2 mg in 2 ml of methanol add 2 ml of water and one drop of sodium hydroxide solution (80 g/litre); orange-red solution turns blue-violet.

PURITY TESTS

1. pH: a 5.0 mg/ml solution in water has a pH of 3.8–6.5 (5) or 4.0–5.5 (4).
2. Water (Karl Fischer): not more than 4.0% (3) or 3.0% (4).
3. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, "Problems on quality assessment of antitumour antibiotics").
4. Limit of content of related substances. No individual related substance should exceed 10 µg/mg estimated as doxorubicin when determined by high-pressure liquid chromatography of 100 µg as described in 6 (b) above.
5. Chemical assay. Determine the absorbance of a 20 µg/ml solution in methanol in a 1-cm layer at the maximum of about 495 nm and calculate the content using an absorptivity value of 22.3; it contains not less than 90% on the basis of the anhydrous substance.
6. HPLC assay (3): it contains not less than 90% on the basis of the anhydrous substance.

BIOLOGICAL ASSAY

Potency: the biological activity may be defined in weight equivalents as in Japan (4) and the United States of America (6).

The potency may be measured by an agar diffusion assay using a suitable test organism, e.g., Bacillus subtilis (ATCC 6633). The potency is between 900 and 1100 microgram equivalents of doxorubicin hydrochloride per mg on the basis of the anhydrous substance.

There is no international standard or chemical reference substance for doxorubicin.
DOSAGE FORM

Doxorubicin is available in an injectable form. It is usual for the dosage form to contain an inert carrier such as lactose or mannitol.

The tests applied to the antibiotic that are also applicable to the dosage form are the following:
1. Physical appearance.
2. Ultraviolet absorption.
3. pH: when reconstituted for use, the pH is 3.8–6.5 (7) or 5.0–6.0 (4).
4. Water (Karl Fischer): not more than 4.0% (4, 7).
5. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, "Problems in quality assessment of antitumour antibiotics").
6. HPLC assay.

ADDITIONAL TESTS FOR THE DOSAGE FORM

1. Identity: it behaves in a manner similar to that of an authentic substance in a thin-layer chromatography test, using silica gel as the coating substance and a mobile phase composed of chloroform: methanol: water in the proportions of 14:6:1.
2. Biological assay: the vial contains 90–115% of the labelled content (7) or 90–125% of the labelled content (4).

REFERENCES

7. United States Code of Federal Regulations, Title 21, Section 450.224 (a) (1).

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MITHRAMYCIN

Synonym: None
Structure:

Produced by: *Streptomyces plicatus*.

IDENTIFICATION TESTS

1. Physical appearance: yellow powder.
2. Ultraviolet absorption. A 0.01 mg/ml solution in methanol exhibits maxima at about 240 nm, 285 nm, 315 nm, and 425 nm, with absorptivity values (1 g/litre, 1 cm) of 18.3, 47.5, 7.2, and 10.5 at the respective maxima (I). A 0.01 mg/ml solution in 0.01 mol/l hydrochloric acid in methanol gives a ratio for the absorbances at the maxima at about 278 nm and 228 nm of 1.96.
3. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of mithramycin per 200 mg of KBr, a spectrum is obtained resembling that of an authentic substance similarly treated. The infrared spectrum (see Appendix) of an authentic sample may be used for comparison.
4. Specific rotation: $[\alpha]_D^{20} -51^\circ$ (c = 0.4 in ethanol) (2).
5. Melting point: 180–183°C (2).
6. Chromatography. The material behaves in a manner similar to that of an authentic substance when examined by thin-layer chromatography, using silica gel as the coating substance, a load of 50 μg, and development with:
(a) a mixture of n-butanol:isobutanol:ethanol:ammonia:water in the proportions of 35:35:15:20:10 (Rf about 0.3), or
(b) a mixture of n-butanol:glacial acetic acid:water in the proportions of 6:2:2 (Rf about 0.73), or
(c) a mixture of n-butanol:acetone:water in the proportions of 30:105:15 (Rf about 0.88), or
(d) a mixture of chloroform:methanol in the proportion of 1:1 (Rf about 0.77), or
(e) a mixture of chloroform:methyl ethyl ketone:ethanol in the proportions of 6:1:4 and detecting with ultraviolet light at 356 nm (Rf about 0.6).
7. Colour tests:
(a) on addition of ferric chloride solution to a methanolic solution of mitramycin an olive-green colour results;
(b) an aqueous solution of mitramycin treated with diazobenzensulfonic acid and sodium hydroxide solution produces an orange-red colour. After boiling with diluted hydrochloric acid, the solution reduces Fehling's solution.

PURITY TESTS

1. pH: a 0.5 mg/ml solution in water has a pH of 4.5–5.5 (3).
2. Loss on drying; not more than 8.0% (3).
3. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, "Problems in quality assessment of antitumour antibiotics").
4. Limit of content of related substances. No related substance found by chromatography identification Test 6 (d).
5. Chemical assay:
(a) determine the absorbance of a 0.01 mg/ml solution in methanol in a 1-cm layer at the maximum at about 283 nm and calculate the content using an absorptivity value (1 g/litre, 1 cm) of 53.7;
(b) determine the absorbance of a 0.01 mg/ml solution in methanol in a 1-cm layer at the maximum at about 420 nm and calculate the content using an absorptivity value of 10.3.
It contains not less than 90% on the basis of the dried substance.

BILOGICAL ASSAY

The biological activity may be defined in weight equivalents of the pure material. The mitramycin reference substance contains 100% of mitramycin (4).

There is no international standard or chemical reference substance for mitramycin.
The potency may be measured by an agar diffusion assay using a suitable test organism, e.g., Staphylococcus aureus (ATCC 6538P). The content is not less than 90% of mitramycin calculated on the basis of the dried substance (2).

DOSEAGE FORM

Mitramycin is available in an injectable form.
It is usual for the dosage form to contain mannitol as a carrier. The tests applied to the antibiotic that are also applicable to the dosage form are the following:
1. Physical appearance.
2. Ultraviolet absorption.
3. Chromatography.
4. pH: when reconstituted for use the pH is 5.0–7.5 (5).
5. Loss on drying: not more than 2.0% (5).
6. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, "Problems in quality assessment of antitumour antibiotics").
7. Limit of content of related substances.
8. Chemical assay (for vial content): it contains 90–110% of the labelled content.

ADDITIONAL TESTS FOR DOSAGE FORM

1. Biological assay: the vial contains 90–110% of the labelled potency (5).
2. Sterility: test by a membrane filtration technique (6).

REFERENCES

4. United States Code of Federal Regulations, Title 21, Section 430.6 (b) (41).

MITOMYCIN

Synonym: Mitomycin C
Structure:

Produced by: Streptomyces caespitosus.

IDENTIFICATION TESTS

1. Physical appearance: blue-violet powder.
2. Ultraviolet absorption. A 0.005 mg/ml solution in methanol exhibits maxima at about 216 nm, 360 nm, and 560 nm, with absorptivity values (1 g/litre, 1 cm) of 74.2, 74.2, and 0.6 at the respective maxima (f).
3. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of mitomycin per 200 mg of KBr, the spectrum obtained resembles that of an authentic substance, similarly treated. The infrared spectrum (see Appendix) of an authentic sample of mitomycin may be used for comparison.

4. Melting point: does not melt below 360 °C (1); decomposes at 360 °C (2).

5. Chromatography: the material behaves in a manner similar to that of an authentic substance when examined by thin-layer chromatography, using silica gel as the coating substance, a load of 100 µg, and development, while protected from light, with:
   (a) methanol (Rf about 0.65), or
   (b) a mixture of chloroform:methanol in the proportion of 9:1 (Rf about 0.21), or
   (c) a mixture of ethyl acetate:acetone in the proportion of 3:2 (Rf about 0.23).

6. Colour tests:
   (a) in acid and alkaline media the colour of the solution changes (1).
   (b) the substance decolorizes solutions of potassium permanganate (2).
   (c) the substance reduces Fehling's solution (2).

PURITY TESTS

1. pH: 5.0 mg/ml in water has a pH of 6.0–8.0 (3) or a saturated solution in water has a pH of 5.5–7.0 (4).
2. Water (Karl Fischer): not more than 5.0% (3).
3. Loss on drying: not more than 2.0% (4).
4. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, "Problems in the quality assessment of antitumour antibiotics").
5. Limit of content of related substances. No related substance found by chromatography Identification Test 5.
6. Chemical assay. Determine the absorbance of a 0.005 mg/ml solution in methanol in a 1-cm layer at the maximum at about 357 nm and calculate the content using an absorptivity value (1 g/litre, 1 cm) of 74.2: it contains not less than 90% on the basis of the anhydrous substance.

BIOLOGICAL ASSAY

1. Potency. The biological activity may be defined in weight equivalents of the pure material as in Japan (4) and the United States of America (3).

   There is no international standard or chemical reference substance for mitomycin.

   The potency may be measured by agar diffusion assay using a suitable test organism, e.g., Bacillus subtilis (ATCC 6633) (3, 4). The minimum potency is 900 microgram equivalents per µg on the basis of the anhydrous substance (3) or 850 microgram equivalents per µg on the basis of the dried substance (4).

DOSAGE FORM

Mitomycin is available in an injectable form, usually containing an inert carrier such as mannitol.
The tests applied to the antibiotic that are also applicable to the dosage form are the following:
1. Physical appearance.
2. Ultraviolet absorption.
3. Chromatography.
4. pH: when reconstituted for use the pH is 6.0–8.0 (5) or 5.0–9.0 (4).
5. Water.
7. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, “Problems in quality assessment of antitumour antibiotics”).
8. Limit of content of related substances.
9. Chemical assay (for vial content): it contains 90–120% of the labelled content.

ADDITIONAL TESTS FOR THE DOSAGE FORM

1. Biological assay: the vial contains 90–120% (5) or 90–125% (4) of labelled potency.
2. Sterility: test by a membrane filtration technique (6).

REFERENCES

5. United States Code of Federal Regulations, Title 21, Section 450.245.
OLOVOMYCIN

Synonym: None
Structure, Olivomycin A:

Produced by: *Streptomyces olivoreticuli*.

**IDENTIFICATION TESTS**

1. Physical appearance: yellow powder.
2. Ultraviolet absorption. A 0.01 mg/ml solution in methanol exhibits maxima at about 225 nm, 275 nm, and 405 nm, with absorptivity values (1 g/litre, 1 cm) of 20.0, 40.0, and 9.5 at the respective maxima (7).
3. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of olivomycin per 200 mg of KBr, a spectrum is obtained resembling that of an authentic substance similarly treated. The infrared spectrum (see Appendix) of an authentic sample of olivomycin A may be used for comparison.
4. Specific rotation: $[\alpha]_D^{19} = 51^\circ$ (c = 0.4 in ethanol) (7).
6. Chromatography. The material behaves in a manner similar to that of an authentic substance, using the following conditions: thin-layer chromatography, a load of 50 µg, silica gel as the coating substance, and development with:
   (a) a mixture of chloroform:methyl ethyl ketone:ethanol in the proportions of 6:1:4, and detection with ultraviolet light at 356 nm ($R_f$ about 1.0);
a mixture of benzene and acetone in the proportion of 1:1 and detection with ultraviolet light ($R_f$ 0.7).

PURITY TESTS

1. pH: a 0.5 mg/ml solution in water has a pH of 4.5–5.5 (3).
2. Water (Karl Fischer): not more than 8.0% (3).
3. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, “Problems in quality assessment of antitumour antibiotics”).
4. Content of olivomycin components. Using a column chromatographic separation by a gradient elution technique followed by the measurement of ultraviolet absorption of separated components, the following limit is required, expressed as percentage of total olivomycin components: olivomycin A not less than 70% (3).
5. Chemical assay. Determine the absorbance of 0.01 mg/ml in 0.01 mol/l hydrochloric acid in methanol in a 1-cm layer at the maximum of about 275 nm and calculate the content using an absorptivity value (1 g/litre, 1 cm) of 41.0; it contains not less than 85% on the basis of the anhydrous substance.

BIOLOGICAL ASSAY

Potency: the biological activity may be defined in weight of the pure material. The olivomycin reference substance contains 100% of olivomycin A.

There is no international standard or chemical reference substance for olivomycin. The potency may be measured by agar diffusion assay using a suitable test organism, e.g., Bacillus subtilis (ATCC 6633). The content is not less than 850 microgram equivalents of olivomycin per mg calculated on the basis of the anhydrous substance (3).

DOSEAGE FORM

Olivomycin is available in an injectable form, usually as the sodium salt without a carrier. The tests applied to the antibiotic that are also applicable to the dosage form are the following:
1. Physical appearance.
2. Ultraviolet absorption.
3. Chromatography.
4. pH: when reconstituted for use the pH is 7.5–8.4 (3).
5. Water: not more than 7.0% (3).
6. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, “Problems in quality assessment of antitumour antibiotics”).
7. Content of olivomycin components.

ADDITIONAL TESTS FOR DOSAGE FORM

1. Biological assay: the vial contains 90–110% of the labelled content (3).
REFERENCES

1. Antitumour antibiotics and their clinical use, Medexport, USSR, p. 41.
3. Instructions for Control of Pharmaceutical Preparation of the Ministry of Health of the USSR.

RUFOCROMOMYCIN

Synonyms: Bruneomycin and streptonigrin.
Structure:

Produced by: Streptomyces flocculus.

IDENTIFICATION TESTS

1. Physical appearance: brown to almost black.
2. Ultraviolet absorption. A 0.01 mg/ml solution in phosphate buffer, pH 7.0, exhibits maxima at about 245 nm and 363 nm, with absorptivity values (1 g/litre, 1 cm) of 80.0 and 26.5 at the respective maxima (1).
3. Infrared absorption. Using a potassium bromide disc containing 1–2 mg of rufocromomycin per 200 mg of KBr, a spectrum is obtained resembling that of an authentic substance similarly treated. The infrared spectrum (see Appendix) of an authentic sample of rufocromomycin may be used for comparison (2).
4. Melting point: 275 °C with decomposition (3).
5. Chromatography. The material behaves in a manner similar to that of an authentic substance, using the following conditions:
   (a) thin-layer chromatography, a load of 50 μg, silica gel as a coating substance, and development with ethyl acetate : benzene : water in the proportions of 3 : 5 : 1 (upper phase);
   (b) paper chromatography using as the mobile phase either acetone : water in the proportion of 1 : 4 or butanol : methanol : water in the proportions of 4 : 1 : 2 and development biologically with Bacillus subtilis (1); a single zone of inhibition is obtained.
6. Colour test. When dissolved in a solution of sodium hydroxide a dark greenish-brown colour is produced which turns red, and ammonia is liberated.

PURITY TESTS

1. Pyrogens, vasodepressor substances, and abnormal toxicity (see section 7, “Problems in the quality assessment of antitumour antibiotics”).
2. Chemical assay. Determine the absorbance of a 0.01 mg/ml solution in phosphate buffer, pH 7.0, in a 1-cm layer at the maximum at about 245 nm and calculate the content using an absorptivity value (1 g/litre, 1 cm) of 80.0; it contains not less than 95% on the basis of the anhydrous substance.
3. Water (Karl Fischer): not more than 8.0% (4).

BIOLICAL ASSAY

1. The content may be defined in weight equivalents as in the USSR (4). The reference standard is 100% pure. There is no international standard or chemical reference substance for rufocomycin.
2. The potency may be measured by agar diffusion assay using a suitable test organism, e.g., Bacillus subtilis (ATCC 6633).
3. The content should be not less than 950 microgram equivalents of rufocomycin per mg on the basis of the anhydrous substance.

DOSAGE FORM

Rufocomycin is available in an injectable and a tablet form, usually as the sodium salt and containing mannitol as a carrier.

The tests applied to the antibiotic that are also applicable to the dosage forms are the following:

1. Physical appearance.
2. Ultraviolet absorption.
3. Chromatography.
4. Pyrogens, vasodepressor substances, and abnormal toxicity, when used for injection (see section 7, “Problems in the quality assessment of antitumour antibiotics”).

ADDITIONAL TESTS FOR DOSAGE FORM

1. Biological assay: the vial contains 90–115% of the labelled content (4).
3. Water (Karl Fischer): not more than 3.0% (4).

REFERENCES

1. Antitumour antibiotics and their clinical use, Medexport, USSR, p. 65.
2. International Centre for Information on Antibiotics (ICIA), No. 13, p. 95.

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6. Reference Materials

(a) National reference materials

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<td>NIH (Japan) Working Standard</td>
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(b) International reference materials

International Reference Preparation of Bleomycin (complex) A5/B5

(c) Standardization of secondary reference materials

National control authorities or manufacturing establishments need supplies of reference materials that can be used on a routine basis.

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1 Available from USP Reference Standards, 12601 Twinbrook Parkway, Rockville, MD 20852, USA. The USP Reference Standard has been designated as the FDA Working Standard (United States Code of Federal Regulations, Title 21, Sections 430.5 (b) and 431.1 (c)).

2 Inquiries concerning the availability of the Working Standards of Japan should be addressed to the Director, National Institute of Health, 10-35, 2-Chome, Kamogawa, Tokyo, Japan.

3 Inquiries concerning the availability of the USSR National Standards should be addressed to the Ministry of Health of the USSR, Moscow, USSR.

4 Available from Rhône-Poulenc-Sané, Direction Recherches, 22 Cours Albert 1er, 75008 Paris, France.

5 Available from the National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, England.
Since the holders of existing national reference materials generally have limited supplies of their primary materials, they cannot be expected to furnish these for routine use by other laboratories. Therefore, there is a need for secondary reference materials (working standards). A working standard is preferably a homogeneous quantity of substance obtained usually from a manufacturer. The substance should be assayed by the procedure referred to in the above sections in terms of an existing national standard. Dosage forms should not be used as secondary reference materials since such preparations may not be homogeneous.

7. Problems in Quality Assessment of Antitumour Antibiotics

(a) Antitumour activity

Since antitumour antibiotics are used in the treatment of cancer, it has been suggested that such substances should be standardized by biological procedures that measure antitumour potency. Although such procedures have been used in testing individual batches of carzinophilin (1), mitomycin (2), and sarcomycin (3) to demonstrate that they possess antitumour activity, the results in general are variable and imprecise. Therefore, it has been usual to demonstrate and measure the biological activity of antitumour antibiotics by assaying their antimicrobial potency.

The antimicrobial activity of these substances is related in only a general way to the antitumour activity. However, antimicrobial assays may be used as quality control measures to ensure the content and consistent quality of batches.

(b) Heterogeneity of antitumour antibiotic preparations

Antitumour antibiotics, like antibiotics used for their antimicrobial properties, are produced by fermentation. Frequently the product of such fermentation is a complex mixture of closely related substances: either a major component with a few minor components, or perhaps two or more major components with a number of minor ones.

For these reasons, methods of quality testing for the first type are intended to confirm that the single major component is present and to limit to a minimum the presence of minor related components. Such determinations generally involve some form of chromatographic separation. These antibiotics are likely to be capable of being charac-
terized by chemical and physical means in such a way as to reflect their biological effect. In testing the second type of mixture it is desirable to know how much of the major components are present and their relative proportions, and at the same time to limit the presence of minor components. These determinations are also generally of a chromatographic nature, but the estimate of biological activity of such mixtures may have to be made by a biological assay. Reference materials are often needed for the calibration of the chromatographic technique and the identification of separated fractions as well as for the biological assays of potency.

(c) Toxicity

Since antitumour antibiotics are generally toxic, some of them have been characterized by means of LD_{50} tests in mice. Some national authorities have used this as an index of the identity and biological activity of batches of certain antitumour antibiotics (dactinomycin (4), doxorubicin (5), mithramycin (6)). Since this test is not usually done in parallel with a reference material, the results obtained may vary owing to changes in animal susceptibility as a result of uncontrolled environmental, nutritional, and other variables. For other antitumour antibiotics, some national authorities have designed "safety" tests or tests for "abnormal toxicity" in which the doses given to the test animals are so low that no deaths are expected with batches of normal quality (actinomycin C (7), bleomycin (8), carzinophilin (9), mitomycin (9)).

(d) Freedom from pyrogens and vasodepressor substances

Since these products are generally prepared in dosage forms intended for parenteral administration, it is necessary that the presence of contaminants such as pyrogens and vasodepressor substances should be held to a minimum. Therefore, in the case of a pyrogen a test must be applied to these dosage forms as part of a quality control programme. Ideally, the dose tested must be selected so as not to affect unduly the wellbeing of the test animals (rabbits). This has not generally been possible. There is a need for research in this area to develop safer and more sensitive tests for pyrogens and indeed for vasodepressor substances, perhaps by devising means of inactivating the toxic property of the antibiotic without inactivating the contaminating pyrogen or vasodepressor substance, if present, or by a preliminary
separation procedure in which the toxic antitumour antibiotic is removed from the solution to be tested.

Another approach is to use the *Limulus* amoebocyte lysate test for endotoxins. This *in vitro* test does not require the use of rabbits but needs further study in relation to its use in the quality assessment of antitumour antibiotics.

A chromatographic separation of endotoxin and bleomycin using a DNA-cellulose column has been described (10). Bleomycin binds to the column allowing endotoxin to be eluted with pyrogen-free water and subsequently tested either in rabbits or by the *Limulus* amoebocyte lysate test. This chromatographic technique may be applicable to the other antitumour antibiotics that form complexes with DNA.

AUTHORS

The Guidelines for Quality Assessment of Antitumour Antibiotics were prepared by the following WHO consultants and staff members:
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Dr J. W. Lightbown, Head, Division of Antibiotics, National Institute for Biological Standards and Control, London, England (Consultant)
Dr F. T. Perkins, Chief, Biologicals, WHO, Geneva, Switzerland
Dr J. D. van Ramshorst, Biologicals, WHO, Geneva, Switzerland
Dr W. W. Wright, Senior Scientist, The United States Pharmacopeia, Rockville, MD, USA (Consultant)

ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice and for supplying additional data relevant to these Guidelines:
Dr V. Davey, Technical Director, Commonwealth Serum Laboratories, Parkville, Victoria, Australia
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Dr R. Netter, Director-General, National Health Laboratory, Paris, France
Dr S. Okamoto, National Institute of Health, Tokyo, Japan
Professor F. Pocchiari, Director-General, Higher Institute of Health, Rome, Italy
Dr C. Puranananda, Bangkok, Thailand
Dr A. van den Bulcke, Standards Laboratory, Brussels, Belgium

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REFERENCES

Appendix

INFRARED SPECTRA OF ANTITUMOUR ANTIBIOTICS

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleomycin</td>
<td>282</td>
</tr>
<tr>
<td>Bleomycin Aβ</td>
<td>283</td>
</tr>
<tr>
<td>Carubicin</td>
<td>284</td>
</tr>
<tr>
<td>Chromomycin Aβ</td>
<td>285</td>
</tr>
<tr>
<td>Dactinomycin</td>
<td>286</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>287</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>288</td>
</tr>
<tr>
<td>Mitramycin</td>
<td>289</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>290</td>
</tr>
<tr>
<td>Olivomycin</td>
<td>291</td>
</tr>
<tr>
<td>Rufocromomycin</td>
<td>292</td>
</tr>
</tbody>
</table>
BLEOMYCIN

2 mg bleomycin/200 mg KBr

[Graph showing absorbance vs. wavelength (μm) and wave number (cm⁻¹)]
BLEOMYCIN A\textsubscript{2}

Approximately 2 mg bleomycin A\textsubscript{2}/200 mg KBr

Wavelength (\textmu m)

<table>
<thead>
<tr>
<th>Wave number (cm\textsuperscript{-1})</th>
<th>Percentage transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1500-1700</td>
<td>60-80</td>
</tr>
<tr>
<td>1800-1900</td>
<td>40-60</td>
</tr>
<tr>
<td>1900-2000</td>
<td>20-40</td>
</tr>
<tr>
<td>2000-2100</td>
<td>10-30</td>
</tr>
<tr>
<td>2100-2200</td>
<td>5-20</td>
</tr>
<tr>
<td>2200-2300</td>
<td>1-5</td>
</tr>
</tbody>
</table>

[Graph showing absorption spectrum with peaks and troughs at various wavelengths and percentage transmission values.]
CARUBICIN

2 mg carubicin/150 mg KBr
CHROMOMYCIN A₃

1.5 mg chromomycin A₃/200 mg KBr

[Graph showing percentage transmission against wavelength (µm) and wave number (cm⁻¹)]
DACTINOMYCIN

1.5 mg dactinomycin/200 mg KBr
DAUNORUBICIN

1.5 mg daunorubicin/200 mg KBr

[Graph with wavelength (μm) on the x-axis and absorbance on the y-axis, showing various peaks and troughs across the spectrum.]
DOXORUBICIN

1.5 mg doxorubicin/200 mg KBr

[Graph showing absorbance versus wave number (cm⁻¹) and wavelength (µm)]
MITHRAMYCIN

1.5 mg mithramycin/200 mg KBr
MITOMYCIN

1 mg mitomycin/200 mg KBr
OLIVOMYCIN

2 mg olivomycin/200 mg KBr

[Graph showing percentage transmission versus wave number (cm\(^{-1}\)) and wavelength (\(\mu m\)).]
RUFOCROMOMYCIN

0.67 mg rufocromomycin/200 KBr
Annex 10

REQUIREMENTS FOR ANTIBIOTIC SUSCEPTIBILITY TESTS\(^1\)

(Requirements for Biological Substances No. 26)
Suggested Changes 1980

INTRODUCTION

Requirements for antibiotic susceptibility tests performed by the agar diffusion technique using antibiotic-impregnated discs were adopted by the WHO Expert Committee on Biological Standardization at its twenty-eighth meeting (WHO Technical Report Series, No. 610, 1977, p. 22). These requirements are also known as International Requirements for Biological Substances No. 26. Part A, section 1.6 of the International Requirements (loc. cit., p. 105) states that “Each disc shall be imprinted with three letters to identify the antibiotic contained therein”. The section also contains a recommendation that the letters “should consist of the first three letters of the International Nonproprietary Name, except when confusion may arise by virtue of the existence of several antibiotics with an identical initial stem or when such letters may be undesirable for other reasons”. A number of such three-letter abbreviations are suggested.

In the case of the cephalosporin antibiotics it is immediately obvious that the first three letters of the International Nonproprietary Name (INN) could not be used because every name begins with “CEF”. Therefore, codes were devised from the initial letter of the name plus two key letters taken from the rest of the name. The following list shows the names of cephalosporin antibiotics with the key letters underlined and the code recommended in the Requirements.

<table>
<thead>
<tr>
<th>INN</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>cefalexin</td>
<td>CEX</td>
</tr>
<tr>
<td>cefaloridine</td>
<td>CFR</td>
</tr>
<tr>
<td>cefalotin</td>
<td>CTN</td>
</tr>
<tr>
<td>cefazolin</td>
<td>CFZ</td>
</tr>
<tr>
<td>cefoxitin</td>
<td>CXT</td>
</tr>
</tbody>
</table>

\(^1\) Prepared by Dr William W. Wright, United States Pharmacopeia, Rockville, MD, USA.
The list also contains other non-cephalosporin antibiotics whose initial letter is “C”:

<table>
<thead>
<tr>
<th>INN</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>carbenicillin</td>
<td>CAR</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>CMP</td>
</tr>
<tr>
<td>clindamycin</td>
<td>CLI</td>
</tr>
</tbody>
</table>

It is noted that two of the three codes employ the first three letters of the INN, while one, that for chloramphenicol, employs the initial letter plus the seventh and eighth letters of the 15-letter name.

Part C, section 4 of the International Requirements includes a directive to use a daily performance test (loc. cit., p. 121) to check the validity of the susceptibility test procedures as performed under conditions prevailing in the particular laboratory. In the performance test, antibiotic discs are tested against reference strains of microorganisms using the same laboratory procedures and conditions used for testing clinical isolates. The performance of each antibiotic disc is evaluated by comparing the diameter of the zone of inhibition on the disc with the range of sizes expected for the particular antibiotic.

The requirements also give, for information purposes, some ranges of zone sizes that have been found appropriate. It is now evident, however, that during the three years since the adoption of the requirements it has become necessary to revise some of the original zone size ranges, and to establish ranges for discs of new antibiotics and for those for which ranges had not yet been determined in 1976.

Other changes have also occurred. As an example, in the case of amikacin, 30-μg discs are now being used in place of 10-μg discs because the 30-μg discs have been found to give more reproducible results.

**SUGGESTED CHANGES**

**Amendment 1**

Part A, section 1.6  Codes

Since 1977 at least three more cephalosporin antibiotics have come into therapeutic use. In considering proposed codes for them, it became obvious that too many abbreviations starting with “C” could
cause confusion. In addition, information became available that the manufacturers of cefoxitin discs had always used the code "FOX". This abbreviation was arrived at by ignoring the first two letters of the name and using the next three letters. It is worth noting that the letter "F" makes an ideal initial letter for cephalosporin antibiotic abbreviations because of the strong "ef" sound in the first syllable of the name of each of them. It is therefore recommended that the present abbreviation for cefoxitin should be changed from CXT to FOX.

Several new cephalosporin antibiotics should be given recommended codes and included in Part A, section 1.6 of the International Requirements. It is recommended that the new abbreviations should start with the letter "F" to distinguish them from the older members of the cephalosporin group, the codes for which start with "C".

<table>
<thead>
<tr>
<th>INN</th>
<th>Proposed code</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>cefamandole</td>
<td>FAM</td>
<td>Three consecutive letters from INN</td>
</tr>
<tr>
<td>cefuroxime</td>
<td>FUR</td>
<td>Three consecutive letters from INN</td>
</tr>
<tr>
<td>cefotaxime</td>
<td>FTA</td>
<td>Name has two a's.</td>
</tr>
</tbody>
</table>

**Amendment 2**

Part C, section 3.8  *Interpretation of results*

Since the impetus for a number of the changes made in the USA can be ascribed to the National Committee for Clinical Laboratory Standards (NCCLS) and its Subcommittee on Antimicrobial Susceptibility Tests, it is recommended that reference should be made to the product of its work in the International Requirements. This reference could properly be made on page 120, in Part C, section 3.8, in the paragraph in small type, by adding the following after "Administration":

"and updated by the National Committee for Clinical Laboratory Standards."

The new footnote would be:

Amendment 3

Part C, section 4.3  Evaluation of performance

The following changes in Part C, section 4.3, of the International Requirements are recommended.

Page 122

Escherichia coli (ATCC 25922)

<table>
<thead>
<tr>
<th>Disc content</th>
<th>Expected range of zone diameters (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>delete:</td>
<td></td>
</tr>
<tr>
<td>amikacin</td>
<td>10 μg</td>
</tr>
<tr>
<td>insert:</td>
<td></td>
</tr>
<tr>
<td>amikacin</td>
<td>30 μg</td>
</tr>
<tr>
<td>cephamandole</td>
<td>30 μg</td>
</tr>
<tr>
<td>cefoxitin</td>
<td>30 μg</td>
</tr>
<tr>
<td></td>
<td>18–24</td>
</tr>
<tr>
<td></td>
<td>19–26</td>
</tr>
<tr>
<td></td>
<td>24–31</td>
</tr>
<tr>
<td></td>
<td>23–28</td>
</tr>
</tbody>
</table>

Page 123

Staphylococcus aureus (ATCC 25923)

<table>
<thead>
<tr>
<th>Disc content</th>
<th>Expected range of zone diameters (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>delete:</td>
<td></td>
</tr>
<tr>
<td>amikacin</td>
<td>10 μg</td>
</tr>
<tr>
<td>insert:</td>
<td></td>
</tr>
<tr>
<td>amikacin</td>
<td>30 μg</td>
</tr>
<tr>
<td>cephamandole</td>
<td>30 μg</td>
</tr>
<tr>
<td>cefoxitin</td>
<td>30 μg</td>
</tr>
<tr>
<td></td>
<td>18–24</td>
</tr>
<tr>
<td></td>
<td>20–26</td>
</tr>
<tr>
<td></td>
<td>28–34</td>
</tr>
<tr>
<td></td>
<td>23–28</td>
</tr>
</tbody>
</table>

Pseudomonas aeruginosa (ATCC 27853)

<table>
<thead>
<tr>
<th>Disc content</th>
<th>Expected range of zone diameters (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>delete:</td>
<td></td>
</tr>
<tr>
<td>amikacin</td>
<td>10 μg</td>
</tr>
<tr>
<td>insert:</td>
<td></td>
</tr>
<tr>
<td>amikacin</td>
<td>30 μg</td>
</tr>
<tr>
<td>polymyxin B</td>
<td>300 IU</td>
</tr>
<tr>
<td></td>
<td>15–22</td>
</tr>
<tr>
<td></td>
<td>18–26</td>
</tr>
<tr>
<td></td>
<td>11–16</td>
</tr>
</tbody>
</table>

Page 127, Appendix 2

Part C, section 3.8 deals with the interpretation of results of antibiotic susceptibility tests on clinical isolates performed using antibiotic discs. Although there has been no international agreement on
categorizing susceptibility, this section cites for information an example (Appendix 2) of a particular set of criteria in wide use.

Some changes and additions have been made by the US Food and Drug Administration and the US National Committee for Clinical Laboratory Standards. It is therefore recommended that the following changes should be made in the table on page 127.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Disc content</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>delete:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amikacin</td>
<td>10 μg</td>
<td>11 or less</td>
<td>12–13</td>
<td>14 or more</td>
</tr>
<tr>
<td><strong>insert:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amikacin</td>
<td>30 μg</td>
<td>14 or less</td>
<td>15–16</td>
<td>17 or more</td>
</tr>
<tr>
<td>cefamandole⁴¹</td>
<td>30 μg</td>
<td>14 or less</td>
<td>15–17</td>
<td>18 or more</td>
</tr>
<tr>
<td>cefoxitin¹</td>
<td>30 μg</td>
<td>14 or less</td>
<td>15–17</td>
<td>18 or more</td>
</tr>
<tr>
<td><strong>delete:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gentamicin</td>
<td>10 IU</td>
<td>12 or less</td>
<td>–</td>
<td>13 or more</td>
</tr>
<tr>
<td><strong>insert:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gentamicin when reporting susceptibility to gentamicin and sisomicin</td>
<td>10 IU</td>
<td>12 or less</td>
<td>13–14</td>
<td>15 or more</td>
</tr>
</tbody>
</table>

Another change recommended in the table on page 127 is the addition of cefaclor and cefadroxil to the list of cephalosporin antibiotics, susceptibility to which can be judged from the results of tests using cefalotin discs.

Page 128, Appendix 2

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Disc content</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>delete:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tobramycin</td>
<td>10 IU</td>
<td>11 or less</td>
<td>12–13</td>
<td>14 or more</td>
</tr>
<tr>
<td><strong>insert:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tobramycin</td>
<td>10 IU</td>
<td>12 or less</td>
<td>13–14</td>
<td>15 or more</td>
</tr>
</tbody>
</table>
Add the following new footnotes:

11 Cefamandole and cefoxitin have a wider spectrum of activity against Gram-negative bacilli than do the cephalosporin antibiotics for which the cefalotin disc is used. Organisms resistant to cefalotin may be susceptible to cefamandole and/or cefoxitin. Therefore, the cefalotin disc cannot be used for testing susceptibility to cefamandole and cefoxitin. Further, the spectra of cefamandole and cefoxitin are dissimilar enough to justify tests using both cefamandole discs and cefoxitin discs.

12 The carbenicillin disc is used for testing susceptibility to carbenicillin and ticarcillin.

The reference to footnote 12 is to be inserted after the two carbenicillin entries in the table.
INTRODUCTION

Biological Substances and their Control

The biological substances referred to here are vaccines and sera for administration to humans. Assurance of their safety and potency requires in-process controls and tests, as well as tests on the final product—procedures necessitating specialized biological knowledge and competence. These substances may be of microbiological origin (vaccines and diagnostic reagents) or of animal or human origin (sera).

The ministry of health is responsible for the suitability of the biological substances used in a community. It is important, therefore, for all such ministries in all countries to establish or recognize pharmacopoeial standards or have access to a means of exercising quality control over biological substances. Such control is generally the
responsibility of a legally constituted national control authority which may differ from one country to another. The authority may be:

(a) the minister of health or other appropriate minister;
(b) a designated national controller; or
(c) the director of a national laboratory concerned with the control of biological products.

The national control authority should be empowered:

(a) to establish criteria or recognize pharmacopoeial requirements for the acceptability of the products;
(b) to establish standard preparations to be used as reference materials in biological assays;
(c) to license manufacturers of biological products, and to license their products for use in the country or for export; and
(d) to establish the technical facilities and other mechanisms needed for implementing the requirements.

The facilities that can be established by a national control authority will depend on the technical and financial resources available. It is important, however, to have some facility, no matter how modest, or to make the necessary arrangements with a country with a comprehensive control system.

In many countries the necessary facilities would have to be created and advice could be obtained from national experts in universities, hospitals, research institutes, public health bodies, or local pharmaceutical companies. Although in some countries controls for food and even for some drugs may exist, the techniques for the control of biologicals differ so markedly from those of chemical analysis that their control will demand a separate facility.

A great need in a developing country is for facilities for the control of immunological products (e.g., vaccines and toxoids, sera and antitoxins, and antivenoms). This Annex therefore deals with the technical arrangements for the separate control of such products.

One of the ways in which WHO assists Member States in the control of immunological substances is through its international biological standardization programme. This programme has two main features:

1. the establishment of international standards and reference preparations for use as reference materials in biological assays, thus permitting the potency of biological products to be expressed in uniform terms (international units or their equivalents) throughout the world;
(2) the provision of sets of international requirements for biological substances, which are intended to provide guidance to those concerned with the production and control of such substances. An important part of this WHO programme is to promote the use and application of international standards and requirements as widely as possible.

Where international standards and reference preparations are needed, these are provided through the Organization’s biologicals programme.

Another way in which WHO can assist Member States is by giving advice on the suitability of products from manufacturing establishments and by reading protocols of the results of in-process and final tests of the manufacture and control of particular batches of vaccine for use in a particular country. WHO is assisted in checking the potency and safety of vaccines and sera by those countries that have comprehensive quality control facilities. Such arrangements can be of immediate assistance to countries that, at the moment, cannot contemplate the establishment of a national control facility, but the aim should be to establish the control of biologicals where feasible.

There are two important reasons why quality control should be established before any manufacture of biologicals is contemplated.

(1) A comprehensive quality control facility can:
(a) check the potency and safety aspects of vaccines on importation, which involves the checking of manufacturers’ protocols, including in-process quality control;
(b) check the potency of vaccines on storage;
(c) check the antibody responses to vaccines; and
(d) carry out serological surveys to check the immunity of a community to infectious diseases.

(2) When a country wishes to become involved in manufacture, which, in the first instance, may consist of the importation of unfinished bulk materials with dilution, blending, filling, and packaging done locally, then the final product must be subjected to quality control. The establishment of a quality control facility, therefore, must precede manufacture.

In some countries the manufacture of biologicals is in progress without any national control, and in some instances there is inadequate testing by the manufacturer. Such situations should be corrected as soon as possible, and steps taken to ensure that only safe and potent vaccines are used in the future.
The national control laboratory furnishes the necessary technical services to the national control authority for the implementation of national regulations relating to biological products. In some cases the director of the laboratory may also be the legally empowered authority.

It is part of the task of the national control authority, in collaboration with the technical staff of the national control laboratory and the manufacturing establishments (if appropriate), to formulate the requirements to be used in a country. In carrying out this task, the international requirements published by WHO and existing national requirements should be taken into consideration. The advisory functions of the laboratory also relate to the various technical questions involved in the application of these requirements. The evaluation of batches of products, based on the interpretation of test results and on other technical information, can be adequately done only if the person making the evaluation has the necessary technical knowledge. The advisory service of the national control laboratory will also provide advice based on the results of the research carried out in the control laboratory for the development of better methods of control.

The release of products may be based on a three-tier system:

1. Products in the production and testing of which there may be inherent dangers (for example, poliomyelitis vaccine and BCG vaccine), or any product from a new manufacturer. These would need batch-by-batch control with the submission of protocols and samples, which may include bulk materials and samples taken during processing, as well as final filled containers.

2. Products for which the test methods are not giving reproducible results or have only just established consistency. These would require the submission of protocols for each batch.

3. Products that have been marketed by the manufacturer for many years without any break in consistency. These could be marketed after the manufacturer had shown the batch to be satisfactory and without further formality.

The scheme outlined in this Annex has been drawn up for the purpose of providing Member States with a guide to the steps that may be followed in providing the technical facilities needed to establish or develop a functioning national control authority for biological substances. The simplest and least expensive step would be to appoint a technical adviser to the national control authority. Although such a
service would have its limitations, it is worth while in countries with no suitable laboratory facilities.

The next step would be to set up laboratory services, though not necessarily with animal facilities since these are very expensive. Much can be achieved by in vitro tests, particularly in checking the maintenance of live virus vaccines that can be used as an index of the continuity of refrigeration facilities (cold chain) and the antibody responses of the community to these vaccines.

The ultimate step is the comprehensive quality control facility that is capable of carrying out all tests on all vaccines and sera.

In some countries the manufacture of biological products is carried out in a governmental institute or one sponsored by the government. Even in such a situation, a national control authority should be established. It may sometimes be the case that, in order to make use of facilities already available (e.g., equipment), manufacture and national control are done in the same establishment. If this is so, the control laboratory should be an independent unit directly responsible to the national control authority (I, pp. 19–22).

**STEP 1**

*Technical Adviser*

At this stage, all that is envisaged is that a competent person should be available to act in an advisory capacity. He may be designated a “technical adviser”.

His advice will be in the form of recommendations to accept or reject particular batches for use, to demand further information, or to require the repetition of tests.

In order to fulfil his functions he should be empowered to:

1. Establish that a product has come from a suitable source. (Advice in this respect may be obtained from WHO or the national control authority of the country of origin.)
2. Ensure that each batch of the product conforms to the relevant international and national requirements, where these exist.
3. Evaluate the protocols of manufacture and testing required from the manufacturer; the WHO requirements include the format of a protocol for the purposes of reporting these data. If the product is manufactured in a country where there is only a technical adviser and
no quality control facilities, the adviser should arrange through WHO for samples to be tested independently. The checking of protocols is a most important part of the control of biological products. It requires meticulous attention to detail and a knowledge of the methods used in the production and control of each of the products under consideration. The information provided in the protocols must make it possible to trace all steps in the manufacture and testing of each batch of a particular product, including all required in-process controls and control tests on the final product. The study of protocols also enables an assessment to be made of the consistency of production of each manufacturer. Such consistency is a great safety factor in the manufacture of biologicals and should be established by all new producers. Before recommending the acceptance of a product from a new source, evidence should be provided that the product is safe, stable, and efficacious.

(4) Inspect samples of the products, their labelling, and accompanying documentation. Visual inspection of a number of samples of each batch may give useful information on the particular product and on the container. The labelling should comply with international and national requirements and enable the batch to be identified in relation to the protocol. The directions on the package inserts must give adequate information on the reconstitution, if appropriate, and mode of administration of the product, recommended storage conditions, and contraindications for use.

(5) Inspect premises and all processes of manufacture including control in the technical adviser’s own country and elsewhere when feasible. This is to verify that the products conform with the general requirements for manufacturing establishments contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (I, p. 11); it also enables independent checks to be made at in-process stages of manufacture. Control activities at this point also include approval of the technical staff manufacturing and testing the products as well as those who are responsible for signing protocols. It cannot be overemphasized that inspection by persons knowledgeable in the manufacture of biologicals contributes greatly to ensuring the quality of biological products. For imported products the technical adviser of the importing country should have the right to demand certificates of conformity with any existing national requirements of the country of origin. Each certificate should be signed by the appropriate government official.

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Much of the information provided to the technical adviser is of a confidential nature and must be treated as such.

**STEP 2**

**Laboratory Facilities for *in vitro* Tests**

Every national control laboratory should deal with biological products manufactured in the country itself as well as those that are imported. The national control laboratory should be autonomous and administered by, or on behalf of, the national control authority, even though, as in some countries, the national control laboratory is housed in a national laboratory that has other functions—e.g., teaching, pathology, diagnostic microbiology, and research.

A limited control laboratory could provide a good measure of technical service for the control of certain biological products.

In addition to the activities previously described, such a laboratory would:

1. Carry out the following control tests:
   1. a) virus titre of live virus vaccines in cell cultures;
   2. b) determination of viable units in BCG vaccine;
   3. c) monitoring of virus titres of live virus vaccines and viable units in BCG vaccine during distribution and use;
   4. d) estimation of antibody content of certain antisera—e.g., diphtheria antibody in cell cultures, and poliomyelitis antibody by neutralization tests in cell cultures;
   5. e) flocculation tests;
   6. f) identity tests; and
   7. g) safety and other tests, as applicable—e.g., protein, residual moisture, and pH.

Sterility testing has been omitted deliberately from this list because, unless a full sterility test is carried out, its interpretation will have little meaning. The inclusion of a full sterility test would markedly increase the size of the staff and facility required. In a limited quality control facility, therefore, it will be necessary to rely for proof of sterility on the manufacturer, whose results will be reported in the protocol, bearing in mind that in-process control of sterility is of greater importance.
(2) Advise the national control authority regarding inspection of manufacturing establishments or, possibly, carry out inspections of such establishments if assigned this responsibility.

(3) Provide some national standards and calibrate them against the appropriate WHO reference materials for antibody content and virus titres.

Details of staff and facilities are given in Appendix 2.

STEP 3

The Comprehensive Facility

For fully comprehensive national control, a laboratory to carry out all essential laboratory and animal tests prescribed in the WHO Requirements must be provided.

Some of the control tests are performed in animals. For this reason the availability of good-quality laboratory animals (guinea-pigs, mice, rabbits) and of a well-equipped animal house are absolutely essential (2).

In addition to those mentioned above, the functions of a comprehensive laboratory should include performance of the following control tests:

(a) Sterility. The tests specified in the revised General Requirements for the Sterility of Biological Substances apply here (3, p. 40).

(b) Identity. Identity tests can be done for many vaccines and sera. These may be based on the morphological appearance of organisms in stained smears or of colonies on growth media. For other vaccines or sera, specific antigens or antibodies are necessary—e.g., for agglutination of pertussis and typhoid vaccines and flocculation of diphtheria and tetanus toxoids.

(c) Innocuity and pyrogenicity. Tests of bacterial vaccines and of most serum products for innocuity require a limited number of guinea-pigs and mice. Tests for pyrogenicity require an adequate supply of rabbits.

(d) Safety. Specific safety tests are carried out on some products, such as diphtheria and tetanus toxoids and pertussis vaccine, using guinea-pigs and mice. With other products such as virus vaccines, more elaborate tests are required, involving the use of monkeys and histological techniques.
(e) Potency. Facilities to carry out all the tests as formulated in the specific WHO Requirements shall be provided. These require an adequate source of good-quality mice, guinea-pigs, and rabbits, as well as fertile hen’s eggs.

(f) Stability. Stability tests based on potency determinations shall be included, particularly for vaccines stored within the country.

A list of the WHO requirements for the specific vaccines and sera is included in Appendix 1. A summary of the appropriate tests for the various vaccines, as far as they are performed in animals or tissues, is given in the accompanying table.

**In vivo and in vitro tests for specific vaccines**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Potency: animal or tissue*</th>
<th>Specific safety: animal or tissue*</th>
<th>General innocuity (guinea-pigs and mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polio (killed)</td>
<td>mo, gp, or other</td>
<td>mo?</td>
<td>+</td>
</tr>
<tr>
<td>Yellow fever</td>
<td>tc</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Cholera</td>
<td>mi</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Smallpox</td>
<td>ra + fe</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Polio (live)</td>
<td>tc</td>
<td>mo + gp + mi + ra + tc</td>
<td>+</td>
</tr>
<tr>
<td>Pertussis</td>
<td>mi</td>
<td>mi</td>
<td>+</td>
</tr>
<tr>
<td>Diphtheria</td>
<td>gp</td>
<td>gp</td>
<td>+</td>
</tr>
<tr>
<td>Tetanus</td>
<td>gp or mi</td>
<td>gp</td>
<td>+</td>
</tr>
<tr>
<td>BCG</td>
<td>vc</td>
<td>gp</td>
<td>+</td>
</tr>
<tr>
<td>Measles</td>
<td>tc</td>
<td>gp + mi + tc</td>
<td>+</td>
</tr>
<tr>
<td>Immune sera</td>
<td>depending on serum titration</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Typhoid</td>
<td>mi + ra</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Influenza</td>
<td>fe</td>
<td>fe</td>
<td>+</td>
</tr>
<tr>
<td>Rabies</td>
<td>mi</td>
<td>—</td>
<td>+</td>
</tr>
</tbody>
</table>
In vivo and in vitro tests for specific vaccines (continued)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Potency:</th>
<th>Specific safety:</th>
<th>General innocuity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>animal or tissue</td>
<td>animal or tissue</td>
<td>(guinea-pigs and mice)</td>
</tr>
<tr>
<td>Meningococcal polysaccharide</td>
<td>1</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Rubella</td>
<td>tc</td>
<td>—</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) te = fertile eggs; gp = guinea-pigs; mi = mice; mo = monkeys; ra = rabbits; tc = tissue culture; vc = viable count.

The only vaccine subjected to tests for pyrogenicity is meningococcal polysaccharide vaccine; immune sera are tested for pyrogenicity.

In addition, provision should be made for research on and development of new and improved methods in quality control.

Details of staff and facilities are given in Appendix 2.

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ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice and for supplying additional data:

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REFERENCES


Appendix 1

LIST OF WHO REQUIREMENTS FOR VACCINES AND SERA*

<table>
<thead>
<tr>
<th>No. of requirement</th>
<th>Year of establishment or last revision</th>
<th>No. in Technical Report Series</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. General requirements</td>
<td>1965</td>
<td>323</td>
</tr>
<tr>
<td>2. Polio (killed)</td>
<td>1965</td>
<td>323</td>
</tr>
<tr>
<td>3. Yellow fever</td>
<td>1975</td>
<td>594</td>
</tr>
<tr>
<td>4. Cholera</td>
<td>1968</td>
<td>413</td>
</tr>
<tr>
<td>5. Smallpox</td>
<td>1965</td>
<td>323</td>
</tr>
<tr>
<td>7. Poliomyelitis (oral)</td>
<td>1971</td>
<td>486</td>
</tr>
<tr>
<td></td>
<td>1980*</td>
<td>658</td>
</tr>
<tr>
<td>8. Pertussis</td>
<td>1978</td>
<td>658</td>
</tr>
<tr>
<td>10. Diphtheria/Tetanus</td>
<td>1978</td>
<td>638</td>
</tr>
<tr>
<td></td>
<td>1980*</td>
<td>658</td>
</tr>
<tr>
<td>11. BCG</td>
<td>1978</td>
<td>638</td>
</tr>
<tr>
<td>12. Measles</td>
<td>1966</td>
<td>329</td>
</tr>
<tr>
<td>14. Human immunoglobulin</td>
<td>1967</td>
<td>361</td>
</tr>
<tr>
<td>15. Typhoid</td>
<td>1967</td>
<td>361</td>
</tr>
<tr>
<td>16. Tuberculin</td>
<td>1968</td>
<td>384</td>
</tr>
<tr>
<td>17. Influenza (inactivated)</td>
<td>1978</td>
<td>638</td>
</tr>
<tr>
<td>18. Immune sera of animal origin</td>
<td>1969</td>
<td>413</td>
</tr>
<tr>
<td>21. Snake antivenins</td>
<td>1971</td>
<td>463</td>
</tr>
<tr>
<td>No. of requirement</td>
<td>Year of establishment or last revision</td>
<td>No. in Technical Report Series</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>22. Rabies (human)</td>
<td>1973</td>
<td>530</td>
</tr>
<tr>
<td></td>
<td>1980</td>
<td>658</td>
</tr>
<tr>
<td>23. Meningococcal polysaccharide</td>
<td>1976</td>
<td>594</td>
</tr>
<tr>
<td></td>
<td>1977&lt;sup&gt;b&lt;/sup&gt;</td>
<td>626</td>
</tr>
<tr>
<td></td>
<td>1980&lt;sup&gt;b&lt;/sup&gt;</td>
<td>658</td>
</tr>
<tr>
<td>24. Rubella</td>
<td>1977</td>
<td>610</td>
</tr>
<tr>
<td></td>
<td>1980&lt;sup&gt;b&lt;/sup&gt;</td>
<td>658</td>
</tr>
<tr>
<td>27. Human blood and blood products</td>
<td>1978</td>
<td>626</td>
</tr>
<tr>
<td>28. Influenza (live)</td>
<td>1979</td>
<td>638</td>
</tr>
<tr>
<td>31. Hepatitis B</td>
<td>1980</td>
<td>658</td>
</tr>
</tbody>
</table>

* These documents may be obtained from WHO on request.
* An addendum that should also be referred to.

**Appendix 2**

**STAFF AND FACILITIES REQUIRED FOR THE CONTROL OF VACCINES AND HUMAN AND ANIMAL SERA**

The size of the staff and the facility will depend on the extent of the quality control contemplated. Advice can be obtained from WHO concerning particular needs. There should be adequate maintenance services and provision for spare parts.

1. **Director or officer in charge (steps 2 and 3); technical adviser (step 1)**

The director or officer in charge (steps 2 and 3) should be a medical or science graduate (or, if appropriate, a graduate in veterinary medicine) with qualifications in microbiology and immunology. The director should have a broad technical knowledge of the production and control of biological substances and should have had one to two years' practical experience in a production and/or control laboratory.

Further periods of laboratory experience may have to be completed when the control of certain biological products is undertaken; this may be necessary also in order to keep abreast of the latest advances in laboratory techniques. Such experience may be gained from time to time by visits to one or more scientists in other countries working in.
the same or related fields so as to exchange ideas, discuss techniques and problems in the work, or analyse the results of studies.¹ The director should be aware of the administrative aspects of control.

A technical adviser (step 1) should have the same qualifications and experience as a director.

2. Scientific officer

The qualifications and experience desirable for a scientific officer are the same as those described above for a director, although the experience required may be limited to a particular control discipline.

3. Technician

A technician should have had a general education up to 16 years of age, and thereafter have obtained a qualification in microbiological techniques (2, 4).

Certain of the special technical skills needed would have to be developed by actual work in the laboratory under the supervision of the higher-qualified staff. It is the responsibility of the director to ensure that his staff receive suitable training.

4. Facilities for the laboratory

(a) Facilities for a step-2 laboratory

As a minimum, a step-2 laboratory should have adequate laboratory space and services (running water, electricity, and/or gas) and some arrangement for working under sterile conditions.

The equipment must include:
— glassware and equipment suitable for general laboratory services;
— an autoclave for sterilizing glassware and media and facilities for membrane-filtration sterilization;
— a refrigerator or other cold-storage facilities, and incubators; and
— equipment and materials for cell-culture procedures.

¹ Assistance can be obtained from WHO (and/or other international agencies such as UNDP) in the form of fellowships, research training grants, and grants for exchanges of research workers. Applications for fellowships should be made to the World Health Organization, Geneva, Switzerland. National governments may also find opportunities for developing consultant services through other international organizations.
(b) Additional facilities for a step-3 laboratory

Arrangements should be made for a regular supply of good-quality animals and for adequate accommodation for them (including facilities for quarantine and conditioning (2)), and possibly for animal-breeding facilities if there are no suitable sources of good-quality animals outside the facility. The extent of the accommodation and the number and species of animals required will depend on the number and variety of tests to be done, including potency tests. Appropriately trained personnel for animal care will also be necessary.

5. Documentation, clerical, statistical, and epidemiological services

In every national control laboratory adequate documentation is essential. This should include at least:

(a) All relevant international requirements for biological substances published by WHO—i.e., general requirements for manufacturing establishments and control laboratories, general requirements for sterility, and requirements for specific products (5, p. 196).

(b) Other relevant specifications and recommendations in WHO publications.\(^2\)

(c) The current national requirements and standard operating procedures of the country itself and of the countries from which biological products are imported.

The laboratory should possess, or have access to, library facilities appropriate to its field of activity.

Suitable clerical facilities are needed for correspondence, preparing reports, issuing certificates and other documents, and keeping accurate records.

Statistical services would be needed if potency tests were being performed. The minimum required would be access to simple cal-

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\(^2\) For example: Specifications for the quality control of pharmaceutical preparations. Second edition of the International Pharmacopoeia (Geneva, World Health Organization, 1967) and the reports of expert committees and other international groups of experts published in the WHO Technical Report Series. Workers and institutions who wish to be kept informed of new reports in these series can do so by regular reference to the summaries of these reports published in the WHO Chronicle. Some further information may also be found from time to time in occasional publications of the Organization. In addition, the Journal of biological standardization (New York, Academic Press) deals with this subject.
culating equipment and to individuals familiar with experimental design and evaluation of data from biological assays.

Since it is essential to confirm both the quality of biological substances and the efficacy of immunization schedules, close cooperation with an epidemiological service is highly recommended.

Annex 12

REQUIREMENTS FOR RUBELLA VACCINE (LIVE)

(Requirements for Biological Substances No. 24)

Addendum 1980

The WHO Expert Committee on Biological Standardization, at its twenty-eighth meeting, adopted the Requirements for Rubella Vaccine (Live), which were annexed to its report (WHO Technical Report Series, No. 610, 1977, pp. 54-87). One section of those Requirements concerns the chromosome monitoring of human diploid cells where such cells are used for the growth of the virus.

Similar sections are to be found in the Requirements for Rabies Vaccine for Human Use (revised) (see pages 81-87) and the Requirements for Poliomyelitis Vaccine, Oral (revised) (see pages 157-168).

There are some differences in the karyology requirements, however, because the more recent requirements were able to take into account the report of the Ad Hoc Committee on Karyological Controls of Human Cell Substrates (Lake Placid, NY, 1978).

As it is desirable for the requirements for chromosome monitoring to be the same for all vaccines produced in human diploid cells, the relevant paragraphs in the Requirements for Rubella Vaccine (Live) have been revised.
Amendment 1

Part A, section 1 Definitions

1.4 Terminology

Add after "Cell seed":

"Manufacturer's working cell bank (MWCB): a quantity of cells derived from a single ampoule of the cell seed and of uniform composition stored frozen at \(-70^\circ\)C or below in aliquots, one of which would be used for the production of each single harvest.

In normal practice such a seed culture (or ampoule) is issued to manufacturers at or near the eighth population doubling level (PDL). This is expanded by serial subculture up to a PDL selected by the manufacturer at which point the cells are combined into one or more pools and preserved cryogenically to form the MWCB. One or more of such ampoules from a pool would be used for the production of a single harvest.

Production cell culture: a collection of cell cultures at the population doubling level used for virus growth that have been derived from a single ampoule of the MWCB."

Amendment 2

Part A, section 3.2.5 Additional test on control cells if human diploid cells are used for production

Replace the whole section by the following:

"3.2.5 Chromosomal characterization and monitoring

3.2.5.1 Chromosomal characterization. At least four samples shall be examined, prepared as described from the cell seed at approximately equal intervals over the life-span of the cell line during serial cultivation. Each sample shall consist of 1000 metaphase cells.

It is also recommended that photographic reconstruction should be employed in the preparation of chromosome-handed karyotypes of 50 metaphase cells per 1000-cell sample using either G-banding or Q-banding techniques. This constitutes 5% of the sample and no specific limits for acceptability are yet recommended. The incidence of karyotypic abnormalities (pseudo-
3.2.5.2 Chromosomal monitoring—preparation and testing. For the determination of the general character of each pool in the manufacturer's working cell bank, a minimum of 500 cells in metaphase shall be examined at the production level or at any passage thereafter for frequency of polyploidy and for exact counts of chromosomes, frequency of breaks, structural abnormalities, and other abnormalities such as despiralization or marked attenuations of the primary or secondary constrictions.

For vaccine production, examination of the cells is usually made between the twenty-seventh and thirty-third population doubling. The national control authority should determine the level of cell population doubling allowable.

For cells examined in metaphase the upper limits\(^1\) of acceptability (upper fiducial limits at 95% (Poisson)) for abnormalities for a 1000- and a 500-cell sample are as follows:

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>1000 cells</th>
<th>500 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatid and chromosome breaks</td>
<td>47/1000</td>
<td>26/500</td>
</tr>
<tr>
<td>Structural abnormalities</td>
<td>17/1000</td>
<td>10/500</td>
</tr>
<tr>
<td>Hyperploidy</td>
<td>8/1000</td>
<td>5/500</td>
</tr>
<tr>
<td>Hypoploidy</td>
<td>180/1000</td>
<td>90/500</td>
</tr>
<tr>
<td>Polyploidy</td>
<td>30/1000</td>
<td>17/500</td>
</tr>
</tbody>
</table>

All cells showing abnormalities shall be subjected to detailed examination, and records shall be maintained of the detailed criteria applied to particular abnormalities evaluated in the karyotype analysis.

Stained slide preparations of the chromosomal monitoring of the working cell bank pool, or photographs of these, shall be maintained permanently as part of the record of the batch of vaccine and for monitoring successive batches made from that cell pool.

It is desirable that a portion of the sample of pooled cell substrate removed from the culture vessels should be stored frozen.

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\(^1\) These upper limits are based on extensive experience with the examination of WI-38 and MRC5 cells reported to and examined by the Ad Hoc Committee on Karyological Controls of Human Cell Substrates, which met in 1978 at Lake Placid, NY, USA. These values will not necessarily be applicable if another human cell strain is used.
so as to retain viability. This would be available for future reference for karyology or for any other purpose relating to the batch of vaccine.

Only those cell pools of the MWCB that have normal karyology shall be used for vaccine production.

3.2.5.3 Identity test of the cells

In some countries, tests for characterizing HLA surface antigens are carried out in addition to chromosome monitoring.”
Annex 13

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

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

Addendum 1980

In the revised requirements (WHO Technical Report Series, No. 638, 1979, pp. 37–115) control tests on final products are listed. One of these tests is the innocuity test. This is performed in mice and guinea-pigs. For diphtheria and tetanus vaccine as well as the DT combined vaccine, at least one human dose, but not more than 5 ml, is injected into guinea-pigs by the intraperitoneal route. For pertussis vaccine and DPT combined vaccine, 5 human doses are injected into guinea-pigs by the intraperitoneal route.

As the innocuity test is intended to be a general safety test of the final product and not a specific toxicity test, the difference in dosage between the two groups of vaccines is not very well founded; moreover, it is generally observed that the injection of 5 ml of these vaccines often results in weight loss or a very low weight gain during the 7 days of the test, which is a cause of concern for producers and control authorities. The requirement is generally considered to be excessive.

For these reasons the following amendment is made to the Requirements as revised in 1978 (Diphtheria, p. 52; Pertussis, p. 73; Tetanus, p. 92).

Section A.5.4  Innocuity test

Replace the whole section by the following:

"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 1 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."

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Annex 14

BIOLOGICAL SUBSTANCES: INTERNATIONAL STANDARDS, REFERENCE PREPARATIONS, AND REFERENCE REAGENTS

The 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. The most recent list was published in 1979 and copies 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.

The Expert Committee, at its thirty-first meeting, made the following changes in the 1979 list:

CORRECTIONS

Page 36 Tuberculin, purified protein derivative (PPD), mammalian

Form in which available
Ampoules containing 10 mg of PPD from a human strain plus 4 mg of salts

Page 42 Human serum proteins, for immunoassay

Transfer to group V on page 46
(This Reference Preparation is held and distributed by the Central Laboratory, Red Cross Blood Transfusion Service, Plesmanlaan 125, Amsterdam, Netherlands. It belongs in group V on page 46.)

Page 56 Interferon, human fibroblast
Interferon, mouse
Interferon, rabbit


318
(These three preparations are not held in London, but are held and distributed by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA.)

Page 70  Delete: Diphtheria toxoid and tetanus toxoid for flocculation tests

Page 71  Delete: Blood group substances A and B
Delete: Bleomycin

Page 72  Delete: Bleomycin AS
Insert: Bleomycin A5
Delete: Tobramycin
Delete: Corticotrophin, human

Page 73  Delete: Desmopressin
Delete: Gonadotrophin-releasing hormone (gonadorelin)

**ADDITIONS**

**Established**

**Antibiotics**

Bleomycin (complex A₂/B₂)  8 910 IU/amp.  First International Reference Preparation 1980
Streptomycin  78 500 IU/amp.  Third International Standard 1980
Tobramycin  986 IU/mg  First International Reference Preparation 1980

(These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England.)

**Antibodies**

Antigens


(These substances are held and distributed by the International Laboratory for Biological Standards, State Serum Institute, 80 Amager Boulevard, Copenhagen, Denmark.)

Blood products and related substances


Human serum complement components 100 IU/amp. (for each component) First International Reference Preparation 1980

(C1q, C4, C5, B)

(These substances are held and distributed by the International Laboratory for Biological Standards, Central Laboratory, Red Cross Blood Transfusion Service, Plesmanlaan 125, Amsterdam, Netherlands.)

Haemoglobin cyanide 10 ml/amp. Second International Reference Preparation 1980

(These substances are held and distributed by the National Institute for Public Health, Postbus 1, 3720 BA Bilthoven, Netherlands.)

Human serum immunoglobulin E 5 000 IU/amp. Second International Reference Preparation 1980

(This substance is held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England.)

Endocrinological and related substances


Desmopressin 27 IU/amp. First International Standard 1980

320
Gonadorelin (gonadotrophin-releasing hormone) for bioassay

31 IU/amp.  First International Reference Preparation 1980

(These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England.)

Reference reagents

Subtype-specific antisera to hepatitis B surface antigen

First International Reference Reagent 1980

(These reagents are held and distributed by the International Laboratory for Biological Standards, Central Laboratory, Red Cross Blood Transfusion Service, Plesmanlaan 125, Amsterdam, Netherlands.)
Annex 15

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>
</table>
| 178 | 1959 | 1. General Requirements for Manufacturing Establishments and Control Laboratories¹  
     |      | 2. Requirements for Poliomyelitis Vaccine (Inactivated)¹ |
| 179 | 1959 | 3. Requirements for Yellow Fever Vaccine¹  
     |      | 4. Requirements for Cholera Vaccine¹ |
| 180 | 1959 | 5. Requirements for Smallpox Vaccine¹ |
| 200 | 1960 | 6. General Requirements for the Sterility of Biological Substances¹ |
| 237 | 1962 | 7. Requirements for Poliomyelitis Vaccine (Oral)¹ |
| 274 | 1964 | WHO Expert Committee on Biological Standardization:  
     |      | 8. Requirements for Pertussis Vaccine¹  
     |      | 9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate³ |

¹ Refer to subsequent revised requirements.
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)

463 1971  WHO Expert Committee on Biological Standardization:
21. Requirements for Snake Antivenins

486 1972  WHO Expert Committee on Biological Standardization:
7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1971)

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1 Refer to subsequent revised requirements.
2 Refer also to subsequent addendum.
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)
23. Requirements for Meningococcal Polysaccharide Vaccine
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)
24. Requirements for Rubella Vaccine (Live)
25. Requirements for Brucella melitensis Strain Rev. 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

WHO Expert Committee on Biological Standardization:

8 and 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Revised 1978)
11. Requirements for Dried BCG Vaccine (Revised 1978)
17. Requirements for Influenza Vaccine (Inactivated) (Revised 1978)
28. Requirements for Influenza Vaccine (Live)

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1 Refer to subsequent revised requirements.
2 Refer also to subsequent addendum.
WHO Expert Committee on Biological Standardization:

7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1971) (Addendum 1980)
22. Requirements for Rabies Vaccine for Human Use (Revised 1980)
23. Requirements for Meningococcal Polysaccharide Vaccine (Addendum 1980)
24. Requirements for Rubella Vaccine (Live) (Addendum 1980)
29. Requirements for Rabies Vaccine for Veterinary Use
30. Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy
31. Requirements for Hepatitis B Vaccine

Guidelines for Quality Assessment of Antitumour Antibiotics

Procedure for Approval by WHO of Yellow Fever Vaccines in Connexion with the Issue of International Vaccination Certificates

Requirements for Immunoassay Kits

The National Control of Vaccines and Sera
Recent reports:

620 (1978) Chemistry and specifications of pesticides
Second report of the WHO Expert Committee on Vector Biology and Control (36 pages) ........................................... 5.—

621 (1978) Epidemiology, etiology, and prevention of periodontal diseases
Report of a WHO Scientific Group (60 pages) .......................... 6.—

622 (1978) The promotion and development of traditional medicine
Report of a WHO meeting (41 pages) ...................................... 5.—

623 (1978) Induced abortion
Report of a WHO Scientific Group (65 pages) ......................... 7.—

624 (1978) Surveillance for the prevention and control of health hazards due to antibiotic-resistant enterobacteria
Report of a WHO meeting (54 pages) ................................. 6.—

625 (1978) Financing of health services
Report of a WHO Study Group (117 pages) .......................... 11.—

626 (1978) WHO Expert Committee on Biological Standardization
Twenty-ninth report (147 pages) ........................................ 14.—

627 (1978) Research in human reproduction: strengthening of resources in developing countries
Report of a WHO Study Group (16 pages) .......................... 4.—

628 (1978) Arterial hypertension
Report of a WHO Expert Committee (58 pages) .................. 6.—

629 (1978) The application of advances in neurosciences for the control of neurological disorders
Report of a WHO Study Group (83 pages) .......................... 9.—

630 (1978) Immunodeficiency
Report of a WHO Scientific Group (80 pages) ................... 7.—

631 (1978) Evaluation of certain food additives and contaminants
Twenty-second report of the Joint FAO/WHO Expert Committee on Food Additives (39 pages) .............................. 5.—

632 (1979) Cancer statistics
Report of a WHO/IARC Expert Committee (47 pages) .......... 5.—

633 (1979) Training and utilization of auxiliary personnel for rural health teams in developing countries
Report of a WHO Expert Committee (35 pages) .................. 5.—
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<th>Description</th>
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<tr>
<td>634</td>
<td>1979</td>
<td>Safe use of pesticides</td>
<td>Third report of the WHO Expert Committee on Vector Biology and Control (44 pages)</td>
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<tr>
<td>636</td>
<td>1979</td>
<td>Controlling the smoking epidemic</td>
<td>Report of the WHO Expert Committee on Smoking Control (87 pages)</td>
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<td>638</td>
<td>1979</td>
<td>WHO Expert Committee on Biological Standardization</td>
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<td>640</td>
<td>1979</td>
<td>WHO Expert Committee on Malaria</td>
<td>Seventeenth report (71 pages)</td>
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<tr>
<td>641</td>
<td>1979</td>
<td>The selection of essential drugs</td>
<td>Second report of the WHO Expert Committee (44 pages)</td>
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<tr>
<td>649</td>
<td>1980</td>
<td>Environmental management for vector control</td>
<td>Fourth report of the WHO Expert Committee on Vector Biology and Control (75 pages)</td>
<td>5.</td>
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655 (1980) Resistance of vectors of disease to pesticides
Fifth report of the WHO Expert Committee on Vector Biology and Control (82 pages) .................................................. 6.—

656 (1981) Assessment of public health and social problems associated with the use of psychotropic drugs

Report of a WHO Scientific Group (76 pages) ..................... 5.—