WHO Expert Committee on Biological Standardization

Thirty-first Report

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World Health Organization, Geneva 1981
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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WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

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$_3$) 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 assayd 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 pro-
thrombin 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 bio-
logical product before it had been licensed in any country. The Com-
mittee 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 investi-
gators 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 trans-
port 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 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).\(^1\) 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,

\(^1\) 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.

ANTIBODIES

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).
BLOOD PRODUCTS AND RELATED SUBSTANCES

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 C1q, 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 C1q, 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 Haemoglobincyanide

The Committee noted that the International Reference Preparation of Haemoglobincyanide, prepared and distributed by the National Institute for Public Health, Bilthoven, Netherlands, had been replaced on 10 occasions since the first Reference Preparation of Haemoglobincyanide 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 Haemoglobincyanide, 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 Haemoglobincyanide 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.

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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/αδ serum
- guinea-pig anti-HBs/αγ serum
- rabbit anti-HBs/αθ serum
- goat anti-HBs/αδ serum
- goat anti-HBs/αγ serum

as International Reference Reagents for the detection of the specific subtypes of hepatitis B surface antigen.
REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

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 Programme 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 Secretariat 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.
Annex 1

PROCEDURE FOR APPROVAL BY WHO OF YELLOW FEVER VACCINES IN CONNEXION WITH THE ISSUE OF INTERNATIONAL VACCINATION CERTIFICATES

Report of a WHO Working Group
Geneva, 22–24 May 1979

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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
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 passage 204.

Since 1937 the 17D vaccine produced in chick embryos has been used on an increasing scale, first in Latin America, and then throughout the world. Initially, multiple passages of 17D virus were maintained 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 encountered in 17D vaccination programmes sponsored by the Rockefeller 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 "innocuous" 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, para-
graph X of the International Sanitary Convention for Aerial Naviga-
tion 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 Navi-
gation, 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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1 Reproduced from: Bonnel, P.H. et al. *Yellow fever vaccination*. Geneva, World
Health Organization, 1956 (Monograph Series, No. 30), pp. 194, 196.
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

<table>
<thead>
<tr>
<th>Sub-cultures</th>
<th>December 1933. Tissue cultures initiated with the virus from the serum of a monkey infected with the unmodified Asibi strain of yellow fever virus.</th>
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<tbody>
<tr>
<td>18th</td>
<td>18 passages in cultures containing mouse embryo tissues, Tyrode solution, and normal monkey serum.</td>
</tr>
<tr>
<td>68th</td>
<td>50 passages in cultures containing whole chick embryo tissues, Tyrode solution, and normal monkey serum (not inactivated).</td>
</tr>
<tr>
<td>204th</td>
<td>136 passages in cultures containing whole chick embryo tissues from which head and spinal cord had been removed, Tyrode solution, and normal human serum (not inactivated).</td>
</tr>
<tr>
<td>221st</td>
<td>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).</td>
</tr>
<tr>
<td>225th</td>
<td>1 chick embryo passage for preparation of vaccine lot No. N.Y.75.</td>
</tr>
<tr>
<td></td>
<td>4 passages in cultures containing chick embryo tissues from which head and spinal cord had been removed, Tyrode solution, and human serum.</td>
</tr>
<tr>
<td></td>
<td>1 passage in chick embryo for preparation of Colombia vaccine No. 70.</td>
</tr>
<tr>
<td></td>
<td>1 passage in chick embryo for preparation of Colombia vaccine Nos. 88 and 90.</td>
</tr>
<tr>
<td></td>
<td>1 passage in chick embryo for preparation of seed virus for vaccine production in New York.</td>
</tr>
</tbody>
</table>

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

180

200

210

220

240

250

260

270

280

290

300

310

320

330

340

350

360

370

380

390

400

---

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.

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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 in 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 (> 10⁹ 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
Dr I. Carter, Epidemiological Surveillance of Communicable Diseases, World Health Organization, Geneva, Switzerland
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

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 (I) 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 inadvisable 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.
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).

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 \(-70{\degree}C\) 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\(_{50}\)): the quantity of a virus suspension that will infect 50\% of cell cultures.

Mouse lethal dose 50\% (mouse LD\(_{50}\)): 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. 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.

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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) (1, 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  Tests 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  Tests 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  Tests 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 mono-specific 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

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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 inocula-
tion 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 in-
tracerebrally. 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 har-
vested 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 neurotoxic
  genic activity becomes demonstrable. This can be done for the animal 
  species and particular strain used for vaccine production by in-
munizing 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 exam-
  ined 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—whichever 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. Ethanolized 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* 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 °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 (11) is used for vaccines made in brains or avian embryos, inclusion of the reference vaccine (see Part A, section 1.3) would enable the sensitivity of the test system to be monitored in the testing of successive batches.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 LD50 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 ± 3°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 virus⁵ to use for the production of vaccine.

The national control authority shall provide or approve the strain for challenge⁴ 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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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 $-70^\circ$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 (MWC): 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 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 (nude 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 (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.

3.1.3.2 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 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>Polyplody</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 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 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 hemadsorbing 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 isoenzymes, HLA, or other immunological tests or karyotype of at least one metaphase spread of chromosomes.

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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
<table>
<thead>
<tr>
<th>Date of preparation of master seed virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of passages between isolation and master seed</td>
</tr>
<tr>
<td>Date of preparation of working seed</td>
</tr>
<tr>
<td>Number of passages between master and working seed</td>
</tr>
</tbody>
</table>

**Virus Production Substrate**

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

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

<table>
<thead>
<tr>
<th>Animal species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult/weanling/suckling</td>
</tr>
<tr>
<td>Number of animals used</td>
</tr>
<tr>
<td>Age of animals</td>
</tr>
<tr>
<td>Quarantine period</td>
</tr>
<tr>
<td>Period between inoculation and harvest</td>
</tr>
<tr>
<td>Result of autopsy</td>
</tr>
<tr>
<td>Result of sterility test on harvested tissue (to be indicated by the terms “pass” or “fail”)</td>
</tr>
</tbody>
</table>

**B. Embryonated eggs**

<table>
<thead>
<tr>
<th>Animal species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin of eggs</td>
</tr>
<tr>
<td>Incubation period</td>
</tr>
<tr>
<td>Period between inoculation and harvesting</td>
</tr>
<tr>
<td>Number of eggs harvested</td>
</tr>
<tr>
<td>Result of sterility test</td>
</tr>
<tr>
<td>Other tests</td>
</tr>
</tbody>
</table>

**C. Cell cultures/avian embryo**

<p>| Amount of cell suspension used in vaccine production |</p>
<table>
<thead>
<tr>
<th>Amount of cell suspension used to prepare control cultures (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results of control tests</td>
</tr>
<tr>
<td>Test for haemadsorbing viruses</td>
</tr>
<tr>
<td>Tests for extraneous agents</td>
</tr>
<tr>
<td>Test for avian leucosis viruses</td>
</tr>
<tr>
<td>Test for adenoviruses</td>
</tr>
<tr>
<td>Other tests (sterility, mycoplasma, etc.)</td>
</tr>
</tbody>
</table>

**D. Cell cultures/human diploid or continuous**

<table>
<thead>
<tr>
<th>Cells used for production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authority by which cell seed was approved</td>
</tr>
<tr>
<td>Amount of cell suspension used in vaccine production</td>
</tr>
<tr>
<td>Amount of cell suspension used to prepare control cultures (ml)</td>
</tr>
<tr>
<td>Results of control tests</td>
</tr>
<tr>
<td>Test for haemadsorbing viruses</td>
</tr>
<tr>
<td>Tests for extraneous agents</td>
</tr>
<tr>
<td>Identity test of cells</td>
</tr>
<tr>
<td>Results of chromosome monitoring of cell seed at production level</td>
</tr>
<tr>
<td>Other tests (sterility, mycoplasma, etc.)</td>
</tr>
</tbody>
</table>

**E. Cell cultures/other cells**

<table>
<thead>
<tr>
<th>Type of cell culture (including host species)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of cell suspension used</td>
</tr>
<tr>
<td>Amount of control suspension investigated (ml)</td>
</tr>
<tr>
<td>Results of control</td>
</tr>
<tr>
<td>Test for haemadsorbing viruses</td>
</tr>
<tr>
<td>Test for extraneous agents</td>
</tr>
<tr>
<td>Test for viruses specific for the host species</td>
</tr>
<tr>
<td>Other tests (sterility, mycoplasma, etc.)</td>
</tr>
</tbody>
</table>
Virus Cultures

Number(s) of culture(s)

Date of inoculation of virus

Date of viral harvest

Bulk Material

Numbers of viral harvests included

Date of pooling

Sterility test

Have all the harvests included been tested for sterility?

Results of these tests

Control of inactivation

Nature of concentration and/or purification (if applied)

Method of inactivation

Date

Temperature

Test for inactivation—volume of material injected (including concentration)

Number of mice injected

Weight of mice

Duration of observation

Other animals (if used)

Result of tests

Result of virus amplification test (for cell vaccines)

Amount of vaccine tested (ml)
### 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. **Inocuity tests**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Guinea-pigs</th>
</tr>
</thead>
</table>
   - Number of animals
   - Route of injection
   - Volume of injection
   - Date of injection
   - Date of end of test
   - Result

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4. **Chemical and biochemical tests**
   - Type of test
   - Result

5. **Potency test**
   - Type of test
   - Date of test
   
   **Immunization of mice**
   - Date of start of test
   - Reference vaccine (potency)
   - Challenge strain
   - Date of challenge
   - ED<sub>50</sub> test vaccine
   - ED<sub>50</sub> reference vaccine
   - Calculated IU/single human dose
   - Confidence limits
   - Results of other potency tests

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

7. **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. 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 important 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 encephalomylitis 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 encephalomylitis 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 immunizations provided that the storage temperature is below \(-60^\circ C\) 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 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 10 passages removed from it.

**Cell seed**: a quantity of cells derived from a single human or animal tissue and of uniform composition, stored frozen at \(-70^\circ C\) 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 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.
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. 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.

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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°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 inoc-
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 leukosis 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.
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-propiolactone (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\(^2\) 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 \(-60^\circ C\) (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

\(^2\) 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 reconstituted 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.

\(^3\) 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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Dr R. Barth, Behringwerke AG, Marburg, Federal Republic of Germany
Dr J. Blameu, Deputy Director, National Centre for Rabies Studies, Malzéville, France
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
| **Period between inoculation and harvesting** |  |
| **Number of eggs harvested** |  |

**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** |  |
| **Tests 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** | 123 |
### Bulk Material

<table>
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<tr>
<th>Description</th>
<th>Information</th>
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<td>Have all the harvests included been tested for sterility?</td>
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<tr>
<td>Result of sterility tests on each viral harvest</td>
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<tr>
<td>Number of viral harvest pooled</td>
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</tr>
<tr>
<td>Nature of concentration and/or purification (if applied)</td>
<td></td>
</tr>
<tr>
<td>Virus titre</td>
<td></td>
</tr>
</tbody>
</table>

### Final Bulk

**Preservatives, etc.**

<table>
<thead>
<tr>
<th>Description</th>
<th>Information</th>
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</thead>
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<tr>
<td>Concentration of antibiotics</td>
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</tr>
<tr>
<td>Stabilizer used</td>
<td></td>
</tr>
<tr>
<td>Result of sterility test on final bulk</td>
<td></td>
</tr>
</tbody>
</table>

### 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
<table>
<thead>
<tr>
<th>Volume of inoculation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of inoculation</td>
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<tr>
<td>Date at end of test</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
</tr>
</tbody>
</table>

**Safety**

<table>
<thead>
<tr>
<th>Animal species used</th>
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</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td></td>
</tr>
<tr>
<td>Volume and route of inoculation</td>
<td></td>
</tr>
<tr>
<td>Date of inoculation</td>
<td></td>
</tr>
<tr>
<td>Date at end of test</td>
<td></td>
</tr>
<tr>
<td>Result</td>
<td></td>
</tr>
</tbody>
</table>

4. **Potency test**

<table>
<thead>
<tr>
<th>Method used</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of inoculation of vaccine</td>
<td></td>
</tr>
<tr>
<td>Minimum titre required for animals for which the vaccine is recommended</td>
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</tr>
<tr>
<td>Titre found</td>
<td></td>
</tr>
</tbody>
</table>

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
Designation and date of establishment of working seed
Number of passages between master and seed

Virus Production Substrate

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

A. Animal neural tissue (brains)
   Animal species
   Quarantine period
   Adult/suckling
   Number of animals used
   Age of animals
   Period between inoculation and harvest
   Result of autopsy

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

127
<table>
<thead>
<tr>
<th>Amount of control cell culture investigated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tests performed on control cultures</td>
</tr>
<tr>
<td>Result</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum for Cell Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin of serum used</td>
</tr>
<tr>
<td>Tests performed on serum</td>
</tr>
<tr>
<td>Result</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Final Bulk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of phenol (if used)</td>
</tr>
<tr>
<td>Concentration of antibiotics used</td>
</tr>
<tr>
<td>Other preservatives (type and concentration)</td>
</tr>
<tr>
<td>Adjuvant (if used)</td>
</tr>
<tr>
<td>Result of sterility test</td>
</tr>
</tbody>
</table>

128
Tests 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**
   - **Abnormal toxicity**
     - Table:
     - Abnormal toxicity
     - Mice
     - Guinea-Pigs
     - Number of animals
     - Route of inoculation
     - Volume of inoculation
     - Date of inoculation
     - Date at end of test
     - Result

   - **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. 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.

130
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 endemic-ally 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 fulminating 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 antignaemic 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 especially 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 vaccine 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 homosexuals, staff and patients in renal dialysis units, inpatients with thalassaemia, and persons whose families include hepatitis B carriers. Other vaccines 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 ay or ad antigens.

Hepatitis B polypeptide vaccines are under development. Furthermore, as there is some evidence that HBV particles have antigenic sites not present on the small particles, vaccines containing viral components 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 absolute 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.
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 antigenaemic 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.

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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
for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (4, p. 49).

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.
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 E₁% ₂₈₀, 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. Immunoelectrophoresis, 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 Sub-
stances 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 anti-
genic content shall be approved by the national control authority.

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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

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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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REFERENCES

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.