WHO Expert Committee on Biological Standardization

Twenty-eighth Report

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WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Geneva, 16–22 November 1976

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WHO EXPERT COMMITTEE
ON BIOLOGICAL STANDARDIZATION

Twenty-eighth Report

The WHO Expert Committee on Biological Standardization met in Geneva from 16 to 22 November 1976. The meeting was opened on behalf of the Director-General by Dr Ch'en Wen-chieh, Assistant Director-General, who observed that much of the agenda of the meeting would be concerned with human blood components and derivatives. A WHO Working Group on the Standardization of Human Blood Products and Related Substances had met in Geneva from 5 to 10 July 1976, and the report of the Group was to be considered in detail as it contained a number of important recommendations. In addition to the establishment of a number of standards and reference preparations, a particular recommendation was the need for the formulation of requirements for the manufacture and control of blood products.

Antibiotics, said Dr Ch'en, were also to be considered and the requirements for antibiotic susceptibility discs would fulfil an urgent need. The increase in antibiotic resistance of organisms was causing concern in many countries and it was essential to be able to compare these findings between countries. The List of Biological Substances had been brought up to date and the substances listed alphabetically within each category in order to facilitate reference. Reference reagents had been reintroduced into the list because of many inquiries concerning the availability of those preparations.

GENERAL

The Committee noted the report of the WHO Working Group on the Standardization of Human Blood Products and Related Substances and considered that the treatment of one particular field in depth by a group of specialists in this manner had been most productive. When necessary, problems in other fields could with advantage be approached in a similar way. The Committee agreed that the report of the Working Group should be annexed to this report (Annex 1).
The use of international units to specify the potency of blood typing sera should be strongly encouraged since international standards have now been established for the four blood typing sera of the greatest practical importance—namely, those for A, B, D and c antigens. The use of the international units will lead to more accurate quantification in the control of potency of blood typing sera, and the use of titres—the traditional way of describing the strength of these sera—should be discouraged. The prophylaxis, monitoring, and treatment of haemolytic disease of the newborn has concentrated particular effort on the quantification of anti-D antibodies. The use of automated instruments for measuring the potency of sera has extended the working dilution range several hundredfold and, as a result, numerical values of endpoint "titres" are cumbersome. The production of sera is now more extensive than ever before, and standardization for their control (for potency, specificity, and stability) is necessary.

The Committee recalled the resolutions of the World Health Assembly that countries should adopt international units to express the potency of biological materials. It is important that this be done widely and rapidly following the establishment of international standards. In those instances where national units differ from the international units the repercussions could be hazardous in clinical practice and cause difficulties in the international exchange of materials and information. It is important, therefore, that national authorities inform WHO of any decision not to use established international units and report the reasons for the decision.

The Fisher-Race nomenclature for Rh blood typing is becoming more widely accepted, and the Committee agreed that it should be universally adopted in the interests of simplicity and uniformity (Annex 1, Part B, section 1). The Committee was informed that studies were in progress to determine whether this system could conveniently be used with computers.

With regard to the collaboration of international scientific organizations with the Expert Committee on Biological Standardization (Annex 1, Part C), the Committee agreed that contact with such specialist organizations could facilitate the rapid dissemination and implementation of WHO recommendations on biological standardization. It could also ensure the availability of specialized help with particular problems and with the establishment of international reference materials. To avoid

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1 See, for example, WHO Handbook of Resolutions and Decisions, Volume II, first edition, 1975, p. 16 (Resolution WHA26.32).
unnecessary duplication of effort it is desirable that scientific organizations planning to undertake work on setting up a proposed international biological standard should keep WHO informed. There has been a steady increase in the number and diversity of substances for which international reference materials are needed, and many of them are being promoted by international societies. To assist such work it had been proposed (Annex 1, Part C) that guidelines be formulated on the methods of setting up international biological standards (including advice on the selection of candidate materials and their processing under suitable conditions), the planning of collaborative studies, tests for long term stability, and the analysis, reporting, and interpretation of results. Such a document could also include guidance on the setting up of national and laboratory standards. The Committee agreed with this proposal and asked WHO to arrange for the formulation of such guidelines.

Standardization for prothrombin time estimations used in the control of anticoagulant treatment with coumarin drugs presents particularly complex problems (Annex 1, Part A, section 6). Standardization became possible only when a way was devised of relating the activity of one preparation of thromboplastin to another using the prothrombin time ratio method. Although this method is based on a procedure that does not conform to the theory of conventional comparative parallel line assays, it has been shown in several studies and in a number of laboratories to give reliable and reproducible results. It is also used extensively for the standardization of thromboplastin preparations. The scheme proposed for international standardization (Annex 1, Appendix 2) depends on the establishment of international reference preparations of certain currently used thromboplastins. Each preparation will have a number assigned to it—called the international calibration constant—to describe its thromboplastin activity relative to a single original reference material for which the value 1.0 has been defined.

These proposals for standardization are based also on the results of extensive studies conducted under the aegis of a joint panel of the International Committee of the International Society of Hematology and the International Committee of the Society of Thrombosis and Haemastasis, modified in the light of comments at a meeting convened by the two bodies in Kyoto, Japan, in September 1976. The proposals allow a choice of schemes for national control of prothrombin time tests and involve the use of an international calibrated scale for expressing the results of prothrombin time estimations. This scheme will have to be explained widely if its introduction into clinical practice is to be successful.
The procedures for calibration of thromboplastins require the use of plasma from patients on long-term coumarin treatment. National authorities should ensure that such plasma is obtained only under controlled conditions and with the knowledge and consent of the patient and his physician.

In view of the current extensive preparation and use of blood products and derivatives both for clinical use and as laboratory reagents, the Committee regarded the recommendation for the formulation of requirements for blood and blood products as particularly urgent.

SUBSTANCES

BLOOD PRODUCTS AND RELATED SUBSTANCES

1. Blood Typing Sera

The Committee noted that the WHO Working Group on the Standardization of Human Blood Products and Related Substances had made a recommendation that national authorities should express potencies of blood typing sera in international units where these are available instead of using titres (Annex 1, Part A, sections 1.1 and 2). The Committee agreed that this was in accordance with resolutions of the World Health Assembly. The Committee noted also the recommendation of the WHO Working Group that general requirements for the production and testing of blood typing sera and ancillary reagents should be formulated. These requirements would include specifications for avidity and specificity, as well as potency, as these are critical characteristics of blood typing sera. The Committee requested WHO to arrange for the formulation of such requirements.

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

The Committee noted that the WHO Working Group on the Standardization of Human Blood Products and Related Substances had expressed the need for reference materials for anti-(A+B), anti-C and

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1 The Expert Committee on Biological Standardization preferred this term to "blood grouping antisera", which was used by the WHO Working Group on the Standardization of Human Blood Products and Related Substances (Annex 1, page 25).

anti-E blood typing sera (Annex 1, Part A, section 1.3). The Committee agreed that such sera were needed and requested the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, to obtain materials suitable for this purpose and to arrange collaborative studies.

The Committee noted also the recommendation that the suitability of the International Standards for Anti-A and Anti-B Blood Typing Sera be reassessed in the light of new knowledge of the ABO system. At the same time, however, it was informed that stocks of the International Standards for Anti-A and Anti-B Blood Typing Sera were depleted. The Committee therefore recommended that the stocks be replaced as soon as possible and requested the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service to obtain materials suitable as replacements and to arrange for collaborative assays.

3. Anti-c Incomplete Blood Typing Serum

The Committee noted the results of the collaborative study of a proposed international standard for anti-c incomplete blood typing serum 76/160, which was coordinated by the Medical Research Council’s Blood Group Reference Laboratory, London, England, and which showed that the preparation was stable. The participating laboratories agreed that the material was suitable for use as an international standard and the WHO Working Group on the Standardization of Human Blood Products and Related Substances endorsed this view (Annex 1, Part A, section 1.2).

The Committee established the preparation as the International Standard for Anti-c Incomplete Blood Typing Serum, Human, and, in agreement with the participants in the study, defined the International Unit for Anti-c Incomplete Blood Typing Serum, Human, as the activity contained in 0.61 mg of the International Standard for Anti-c Incomplete Blood Typing Serum, Human.

4. Anti-D Immunoglobulin

The Committee noted the advice of the WHO Working Group on the Standardization of Human Blood Products and Related Substances concerning the suitability of a freeze-dried preparation 68/419 to serve as an international reference preparation of anti-D immunoglobulin (Annex 1, Part A, section 2). An international study involving 17 laboratories in 11 countries had shown that the isotopic method of determining anti-D immunoglobulin gave results similar to those produced by the
manual and automated haemagglutination techniques. The material had been calibrated in international units by 11 laboratories, its activity having been compared with that of the International Standard for Anti-Rh6 (anti-D) Incomplete Blood Typing Serum. This study showed that the immunoglobulin preparation had an activity of 150 IU per ampoule. The Committee established the preparation as the International Reference Preparation of Anti-D Immunoglobulin and noted that each ampoule contained 14.76 mg of the preparation (150 IU per ampoule).

The Committee agreed that any future replacement of the International Reference Preparation of Anti-D Immunoglobulin should also be assigned a unitage by comparison with the International Standard for Anti-Rh6 (anti-D) Incomplete Blood Typing Serum.

5. Anti-Hepatitis B Immunoglobulin

The Committee noted that the WHO Working Group on the Standardization of Human Blood Products and Related Substances had reported that an international reference preparation was needed for the estimation of hepatitis B antibody (Annex 1, Part A, section 8.1). A batch of human anti-hepatitis B immunoglobulin that might be suitable for this purpose would be examined in a collaborative study organized by WHO. The Committee was informed that immunoglobulin from subjects immunized with an inactivated hepatitis B vaccine could also be made available and agreed that such a preparation should be included in the study. The Committee observed that there was an urgent need for such an international reference preparation.

6. Anti-Varicella Zoster Immunoglobulin

The Committee noted that the WHO Working Group on the Standardization of Human Blood Products and Related Substances had recommended the setting up of an international reference material for anti-varicella zoster immunoglobulin (Annex 1, Part A, section 8.2). The Committee supported this recommendation and asked the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, to obtain a suitable preparation.

7. Blood Coagulation Factor VIII

The Committee noted (Annex 1, Part A, section 3.1) that the first International Standard for Blood Coagulation Factor VIII had been established before sensitive tests for hepatitis B surface antigen (HBsAg)
had been developed. The material was later found to give positive results in tests for HBsAg and its continued distribution was therefore considered undesirable. A replacement material shown to be free from HBsAg had been subjected to collaborative assay by 17 laboratories in 13 countries and had been shown to be suitable.

The Committee authorized the National Institute for Biological Standards and Control, London, to establish the preparation as the second International Standard for Blood Coagulation Factor VIII on the basis of the results of the collaborative assay and, with the agreement of the participants in that assay, to define the international unit.

8. Blood Coagulation Factor IX

The Committee noted the statement of the WHO Working Group on the Standardization of Human Blood Products and Related Substances that there was an urgent need for an international standard for blood coagulation Factor IX. The Committee noted also the results of a collaborative study in which a proposed international standard for blood coagulation Factor IX concentrate (72/32) had been compared with samples of fresh normal plasma, with freeze-dried pooled plasma, and with another Factor IX concentrate (Annex 1, Part A, section 3.2).

On the basis of the results of the collaborative assay the Committee established the proposed standard as the International Standard for Blood Coagulation Factor IX and defined the International Unit for Blood Coagulation Factor IX as the activity contained in 1.05338 mg of the International Standard for Blood Coagulation Factor IX.

9. Plasmin

The Committee noted that the WHO Working Group on the Standardization of Human Blood Products and Related Substances had recommended that preparation 72/379 of partially purified plasmin in aqueous glycerol solution, which had been shown in a collaborative assay to be stable at 4°C, should be considered for use as an international reference preparation (Annex 1, Part A, section 4.1). In view of the urgent need for a plasmin reference preparation, the Committee established this preparation as the International Reference Preparation of Plasmin and defined the International Unit of Plasmin as the activity contained in 0.125 ml of the International Reference Preparation of Plasmin.

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In view of the instability of the International Reference Preparation above 20°C the Committee agreed that each ampoule distributed should have a temperature-sensitive indicator attached to it with an instruction that the preparation should not be used if the indicator shows that the temperature has exceeded 20°C.

10. Thromboplastins

The Committee noted that the WHO Working Group on the Standardization of Human Blood Products and Related Substances had recommended the need for an international reference preparation of each of four common types of thromboplastin currently used for standardization in prothrombin time estimations (Annex I, Appendix 2).

The Committee noted also that a preparation of each of these types had been included in international collaborative studies in which they had been related to each other in prothrombin time tests on normal and coumarin plasmas. The results of these studies had shown them to be stable and suitable to serve as reference materials.

The Committee therefore established preparation NIBSC 67/40 as the International Reference Preparation of Thromboplastin (Human, Combined) and defined its International Calibration Constant as 1.0. The Committee noted the recommendations of the WHO Working Group that preparation NIBSC 68/434—the proposed international reference preparation of thromboplastin (bovine, combined)—should have assigned to it an international calibration constant of 1.0; preparation NIBSC 69/223—the proposed international reference preparation of thromboplastin (human, plain)—should have assigned to it an international calibration constant of 0.90; and preparation NIBSC 70/178—the proposed international reference preparation of thromboplastin (rabbit, plain)—should have assigned to it an international calibration constant of 0.60.

The Committee was informed that the three values suggested as calibration constants were based on the results of an international collaborative study involving more than 200 laboratories. Since the report of this study has not reached a final form, the Committee requested WHO to communicate the completed report to the members of the Expert Advisory Panel on Biological Standardization, so that the Director-General could be asked to establish the international reference preparations and to assign to them the appropriate international calibration constants.
The provision of four preparations each with its own calibration constant is necessary because calibration is more precise (i.e., the standard deviation of the slope of the line is less) when a preparation of thromboplastin is calibrated against a reference thromboplastin of the same type rather than against one of a different type. The Committee agreed that any replacement of these proposed preparations, or any reference thromboplastin of another type that may be established as an international reference preparation, must be assigned a calibration constant in terms of the International Reference Preparation of Thromboplastin (Human, Combined), which is the only one for which a constant has been defined.

The Committee requested WHO to formulate guidelines for national authorities on how the international calibrated scale can be brought into use at the national level (see Annex 1, Appendix 2, recommendation 4).

11. Ancrod

The Committee noted that the WHO Working Group on the Standardization of Human Blood Products and Related Substances had agreed that an international standard for ancrod was required (Annex 1, Part A, section 5.1). A purified preparation, 74/581, had been freeze-dried at the National Institute for Biological Standards and Control, London, and with the agreement of manufacturers had been established as the British Standard for ancrod with an activity of 55 units per ampoule. Part of this preparation was available for use as an international reference preparation.

The Committee, therefore, established this material as the International Reference Preparation of Ancrod, adopted the national unit and defined the International Unit for Ancrod as the activity contained in 0.307 mg of the International Reference Preparation of Ancrod.

12. Fluorescein Isothiocyanate-Conjugated Sheep Anti-Human Immunoglobulin

The Committee noted the report on the collaborative assay of a freeze-dried FITC-conjugated sheep anti-human immunoglobulin \(^1\) (preparation SBL 480010) jointly arranged by WHO and the International Union of Immunological Societies. The Committee was informed that the report had been communicated to the members of the

\(^1\) Unpublished working document WHO/BS/76.1127.
Expert Advisory Panel on Biological Standardization and other experts and that, as no adverse comments had been received, the Director-General had established this preparation as the International Standard for FITC-Conjugated Sheep Anti-Human Ig. On the basis of the results of the collaborative assay and with the agreement of the participants, the Director-General had defined the International Unit for FITC-Conjugated Sheep Anti-Human Ig.

**ANTIBIOTICS**

13. **Candidin**

The Committee noted that the collaborative assay of candidin requested in its twenty-seventh report \(^1\) was now almost complete but that it had not been possible to include a sample of levorin \(^2\) as requested.

14. **Bleomycin and Doxorubicin**

The Committee was informed of the progress being made by the WHO Secretariat in drafting international specifications for bleomycin and doxorubicin.

The draft specifications indicated a probable need for chemical reference preparations and a possible need for biological reference preparations. The Committee was informed that a single preparation of each antibiotic might serve both chemical and biological purposes.

15. **Spectinomycin**

The Committee noted that in accordance with the authorization in its twenty-seventh report \(^3\) the National Institute for Biological Standards and Control, London, on the basis of the results of the collaborative assay \(^4\) and with the agreement of the participating laboratories had defined the International Unit for Spectinomycin as the activity contained in 0.00149 mg of the International Reference Preparation of Spectinomycin.

\(^2\) Unpublished working document WHO/BS/75.1131.
\(^4\) Unpublished working document WHO/BS/76.1134.
16. Tobra mycin

The Committee noted that in accordance with the request in its twenty-seventh report, the National Institute for Biological Standards and Control, London, had obtained material suitable to serve as an international reference preparation of tobramycin and that a collaborative assay would be arranged.

17. Ristocetins

International Reference Preparations of Ristocetin and of Ristocetin B were established in 1960 and 1964 respectively. The Committee, in its eighteenth report, noted that ristocetin was no longer produced and concluded that the collaborative assay that had been planned in order to define an international unit was no longer necessary. The manufacture of this antibiotic has not been resumed, and since there is no longer a need for the definition of the activity of this antibiotic the Committee discontinued the International Reference Preparation of Ristocetin and the International Reference Preparation of Ristocetin B.

ANTIGENS

18. Diphtheria Toxoid, Adsorbed

The Committee noted that as requested in its twenty-seventh report the Statens Serum Institut, Copenhagen, had arranged a collaborative study, in which nine laboratories had taken part, for the replacement of the International Standard for Diphtheria Toxoid, Adsorbed.

19. Tetanus Toxoid, Adsorbed

The Committee noted that stocks of the International Standard for Tetanus Toxoid, Adsorbed, were depleted and that a replacement would be needed. The Committee agreed that a purified preparation

6 Unpublished working document WHO/BS/76.1133.
7 Unpublished working document WHO/BS/76.1135.
8 Unpublished working document WHO/BS/76.1137.
similar to those routinely produced should be used for this purpose. The Statens Seruminstitut, Copenhagen, had prepared a purified tetanus toxoid that had been adsorbed on to aluminium hydroxide and stabilized for freeze-drying. The first preparation (TESA 52) proved to be unsuitable and a further preparation will be made for the replacement.

20. Tetanus Toxin

The Committee noted that the WHO Working Group on the Standardization of Human Blood Products and Related Substances had expressed the need for a tetanus test toxin for common use in assays of antitoxin content in reference preparations (Annex 1, Part A, section 8.3). The WHO Secretariat had contacted a number of laboratories that could provide toxin and could take part in a collaborative study and had requested the National Institute for Biological Standards and Control, London, to coordinate the collaborative study.

The Committee agreed that, in spite of the technical difficulties that had previously prevented international agreement on the adoption of a single preparation, an attempt should be made to test the present feasibility of such a proposition.

21. Carcinoembryonic Antigen

The Committee noted that in accordance with the request in its twenty-seventh report the WHO Secretariat in conjunction with the National Institute for Biological Standards and Control, London, had made inquiries about the units that might have been assigned to preparations of carcinoembryonic antigen in use in various countries. It was confirmed that the unitage assigned to the International Reference Preparation of Carcinoembryonic Antigen was not in conflict with the definition of any other preparations of this material.

22. Purified Protein Derivative of Bovine Tuberculin
   (for Veterinary Use)

The Committee noted that, as requested in its twenty-seventh report, the Central Veterinary Laboratory, Weybridge, England, had prepared a batch of purified protein derivative (PPD) of bovine tuberculin that

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1 Bulletin of the League of Nations, 10: 104-143 (1942).
4 Unpublished working document WHO/B5/76.1136.
was considered suitable to serve as an international standard. A collaborative assay involving six laboratories within the European Economic Community had been completed and a wider international collaborative assay was being arranged. The Committee was informed that tests would be carried out in sensitized guineapigs and infected cattle.

ANTITOXINS

23. *Clostridium botulinum* Type B Antitoxin

The Committee noted that stocks of the International Standard for *Clostridium botulinum* Type B Antitoxin were depleted and that a replacement would be needed before the studies on the heterogeneity of the *C. botulinum* type B toxins referred to in the twenty-seventh report could be started. The Committee noted that preliminary tests with a purified *C. botulinum* type B antitoxin, prepared as the replacement, did not give widely different potencies when titrated with the toxins prepared from proteolytic and non-proteolytic strains. Such differences were much less than those shown in the report noted by the Committee at its twenty-seventh meeting.

The Committee requested the Statens Seruminstitut, Copenhagen, to obtain a sufficient quantity of the material, to process it as a proposed international standard, and to arrange for a collaborative assay as well as to initiate various studies in conjunction with the Chiba Serum Institute, Japan, using *C. botulinum* type B toxins prepared from both proteolytic and non-proteolytic strains.

REAGENTS

24. Adenovirus Antisera Types 4, 19, 20, 22, 23 and 24

The Committee noted that tests on the antisera prepared by the Center for Disease Control, Atlanta, USA, as reference reagents for adenovirus types 4, 19, 20, 22, 23 and 24 had shown that they were specific and stable.\(^2\) The data had been sent to members of WHO's

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\(^1\) Unpublished working document WHO/BS/76.1139.
\(^3\) Unpublished working document WHO/BS/76.1133.
Expert Advisory Panel on Biological Standardization and no adverse comments had been received. The Committee therefore established the antisera as the International Reference Reagents of Adenovirus Antisera Types 4, 19, 20, 22, 23 and 24.

REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

25. Requirements for Red Blood Cells for Immunization

The Committee noted that the WHO Working Group on the Standardization of Human Blood Products and Related Substances had reviewed the recommendations of a WHO scientific group concerning the immunization of donors to obtain highly potent anti-D plasma for anti-D immunoglobulin (Annex 1, Part A, section 1.5). These recommendations of the WHO Scientific Group on the Prevention of Rh Sensitization were formulated in 1970, when less was known about hepatitis and the use of frozen cells was not normal practice. The Committee therefore asked WHO to arrange for the revision of the recommendations for the immunization of donors and to formulate specifications for reagent red blood cells used for compatibility tests.

26. Requirements for the Processing and Control of Human Blood and its Derivatives

The Committee noted the statement of the WHO Working Group on the Standardization of Human Blood Products and Related Substances that there was an urgent need for international requirements for the processing and control of human blood and its derivatives (Annex 1, Part B, section 2).

The Committee asked WHO to arrange for the formulation of such requirements, which should cover all procedures from the collection of blood to the quality control of the final products.

27. Requirements for Meningococcal Polysaccharide Vaccine

The Committee was informed that amendments to the Requirements for Meningococcal Polysaccharide Vaccine had been found to be necessary owing to advances in technology, which now enabled poly-

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saccharides of higher molecular weight and greater purity to be made. The amendments, which had been prepared by the WHO Secretariat in collaboration with a number of experts, were adopted by the Committee as an Addendum to the Requirements (see Annex 2).

28. Requirements for Rubella Vaccine (Live)

The Committee noted that in accordance with the request in its twenty-seventh report the proposed requirements for rubella vaccine had been prepared.

The Committee noted that vaccines prepared from different strains of rubella virus and attenuated in different cell cultures are now considered to be safe for general use although they still produce mild reactions in some subjects. The Committee noted also that the requirements differ from earlier requirements for virus vaccines in their insistence on control of the colony of animals, flock of birds, or seed stock of a cell strain—a requirement that increases safety with respect to freedom from extraneous agents. When testing the cell cultures for freedom from such agents, therefore, the samples are taken late in the growth of the cell cultures rather than at the time the production cultures are inoculated with virus. The Committee noted that this is the first occasion on which tests to show freedom from particular extraneous agents and inhibitors are specified in WHO requirements for the serum used for the growth of cell cultures.

After making a number of minor amendments to the document, the Committee adopted the Requirements for Rubella Vaccine (Live) and agreed that they should be annexed to this report (Annex 3).

29. Requirements for Brucella melitensis Strain Rev. 1 Vaccine (Live—for Veterinary Use)

The Committee noted that in accordance with the request in its twenty-seventh report the proposed requirements for Brucella melitensis strain Rev. 1 vaccine (live—for veterinary use) had been communicated to members of the Expert Advisory Panel on Biological Standardization as well as to producers of the vaccine and other experts in the subject. The comments received had been taken into consideration in preparing the revised document.

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The Committee agreed that in view of the pathogenicity of B. melitensis for humans, who may become infected from sheep or goats, the immunization of these animals for eradication of the disease is important. The B. melitensis strain Rev. 1 has been shown to be less virulent for animals, and the requirements specify tests to ensure that the vaccine is made from the attenuated strain. Since B. melitensis strain Rev. 1 has been shown to dissociate unless care is taken in the growth of the organism, the requirements include a test for dissociation as a precaution against making the vaccine from the dissociated strain.

After making minor amendments, the Committee adopted the Requirements for Brucella melitensis Strain Rev. 1 Vaccine (Live—for Veterinary Use) and agreed that they should be annexed to this report (Annex 4).

30. Requirements for Antibiotic Susceptibility Discs

The Committee noted that in accordance with the request in its twenty-seventh report, requirements for antibiotic susceptibility discs had been prepared. The Committee discussed the proposed requirements and agreed that they would be most helpful especially to those countries where there is no control over such antibiotic discs. Although there are a number of tests for measuring the susceptibility of microorganisms to antibiotics the one in most common use is the diffusion of the antibiotic from a disc placed on agar inoculated with the microorganism being tested; the requirements are applicable only to this technique. The details of the technique are so critical that the requirements include an example of directions for use of the antibiotic susceptibility discs as well as specifications for the agar medium used in performing antibiotic susceptibility tests. Furthermore, the appendices of the requirements include a sample calculation for an assay of the content of a disc and an example of the criteria for interpretation of zone size data obtained in susceptibility tests.

The Committee adopted the Requirements for Antibiotic Susceptibility Tests—I. Agar Diffusion Tests using Antibiotic Susceptibility Discs and agreed that should other well controlled techniques become common practice then other requirements applicable to such techniques should be drafted. The Committee agreed that the Requirements should be annexed to this report (Annex 5).

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31. List of Biological Substances

The Committee noted that, in accordance with the suggestion in its twenty-seventh report, the list of biological substances has been redrafted. The substances formally included under hormones, vitamins and enzymes have been reclassified, and blood products and related substances are now placed in a new section, as are endocrinological and related substances. All substances within each category are now listed in alphabetical order, and the list of reference reagents, which was omitted from the 1975 list, has been reintroduced.

The Committee considered that the present format was an improvement and agreed that any additions to the list should, as in the past, be annexed to the Expert Committee report until sufficient additions have accumulated to make reprinting of the document worthwhile. The Committee recommended, however, that the list annexed to each successive report should be cumulative until such time as the list is reprinted. The possibility is being explored of having the list prepared as a computer printout, but at the moment this did not appear to be practical.

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2 For further information see Annex 7.

ACKNOWLEDGEMENTS

The Committee wished to record its thanks to the following members of the WHO Secretariat for their special contributions to its deliberations: Dr F. Lothe, Health Laboratory Technology, and Dr W. Wieniawski, Pharmaceuticals.
Annex I

REPORT OF A WHO WORKING GROUP ON THE STANDARDIZATION OF HUMAN BLOOD PRODUCTS AND RELATED SUBSTANCES

Geneva, 5–10 July 1976

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1 Only those parts of the report relevant to the work of the WHO Expert Committee on Biological Standardization are included in this annex. The full report is contained in unpublished working document WHO/BS/76.1130, which is available to persons professionally interested in the subject from Biological Standardization, World Health Organization, 1211 Geneva 27, Switzerland.

² The WHO Expert Committee on Biological Standardization preferred the term "blood typing sera" to "blood grouping antisera" since it had been used in all previous reports of the Committee.

³ The WHO Expert Committee on Biological Standardization preferred the terminology Anti-(A + B).

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PART A:
SUBSTANCES

1. STANDARDIZATION IN BLOOD GROUP SEROLOGY

1.1 Blood grouping antisera: general

Standardization in blood group serology was first required when blood typing sera were used for the classification of red cell phenotypes. It has been common practice to express the strength of these antisera in terms of titres, but these may vary widely depending on such factors as red cell age, phenotype and concentration, genetically determined variations in cells and immunoglobulins, ionic strength and pH of the test milieu, additives, incubation time, temperature, force and time of centrifugation, resuspension technique, and the assessment of the endpoint. Any of these conditions may vary markedly from laboratory to laboratory, but the effect of these variables can be reduced if the antisera used have been calibrated in comparative assays against national standards labelled with international units.
The Working Group noted that international standards for anti-A and anti-B blood typing sera had been established in 1950 and that a standard for anti-Rh₀ (anti-D) incomplete had been established in 1966. The establishment of a standard for anti-c, therefore, would satisfy the need for standards for the most important blood grouping antisera. It was suggested that national specifications for the characterization of these antisera should include the determination of potency in comparative assays with national standards calibrated in international units. This was particularly important for the national control of the production of reagents in order to ensure that they have satisfactory potency from their date of issue until their expiry date. The Group noted that specificity and avidity are also critical performance characteristics of blood grouping antisera and agreed that they should be covered by specifications. Such specifications should provide a margin of safety when the reagents are used under the test conditions specified.

The Group urged WHO to encourage national authorities to express potencies in international units where available and to discourage the use of titres for this purpose.

1.2 Anti-c

The Group noted that a proposed international standard for anti-c incomplete blood typing serum (coded 67/160) had been prepared, had been shown to possess satisfactory stability, and had given satisfactory results when subjected to an international collaborative study carried out by the WHO International Blood Grouping Reference Laboratory and the National Institute for Biological Standards and Control, London. The participating laboratories agreed that the proposed material was suitable for use as an international standard, and agreed on the assignment of a unitage of 64 IU per ampoule to the material. The Group recommended that WHO take steps to establish the material 67/160 as the International Standard for Anti-c Incomplete Blood Typing Serum with the unitage agreed by the participants in the collaborative study.

1.3 Anti-A, B, anti-C, Anti-E, and anti-e

The Group noted that no WHO requirements or candidate reference materials exist for blood grouping antisera anti-A, B, anti-C, anti-E or anti-e. The Group considered (1) that anti-A, B is useful for the detection of some weak subgroups of A that may be missed with anti-A sera as well as for checking the results of routine anti-A and anti-B tests, particularly in automated systems; (2) that anti-C and anti-E are needed
for Rh phenotyping of patients as well as for subtyping Rh negative donors; and (3) that anti-e serves a less important clinical role. The Group recognized the need to standardize these and other blood grouping reagents with regard to potency, specificity, and avidity in order to ensure that their performance in routine blood bank operations is satisfactory.

The Group recommended that the WHO Secretariat draw up general requirements for the production and testing of blood grouping antisera and other ancillary reagents, such as anti-human globulin and bovine albumin. Candidate reference preparations should then be obtained, evaluated for stability and, if found suitable, subjected to international collaborative studies.

The Group recommended also that the suitability of the International Standards for Anti-A and Anti-B Blood Grouping Sera be reassessed in the light of new knowledge of the ABO system acquired since the standards were established.

Finally the Working Group considered the feasibility of preparing candidate reference materials for "complete" anti-D grouping sera. As these antibodies are in many cases of the IgM type special attention should be paid to the stability of the preparations.

1.4 Anti-human globulin

There is widespread agreement that specifications and reference materials are needed for anti-human globulin (Coombs's) reagent, and the Group noted that work towards these objectives has been in progress for several years by a Working Party on the Standardization of Anti-globulin Reagents under the auspices of the International Committee for Standardization in Hematology (ICSH) and the International Society for Blood Transfusion (ISBT).

The ICSH/ISBT Working Party has defined the requirements for a polyspecific antiglobulin reagent to be used in compatibility testing as follows.

1. The reagent should contain anti-IgG activity, which must be such that red cells weakly sensitized by non-complement binding antibodies of different specificities are agglutinated by the reagent; anti-D, anti-K, anti-Fy*, and anti-Jk* should be used as a minimum in testing the reagent.²

² The Group noted that some modifications of these requirements might be appropriate for non-Caucasian populations, especially with respect to anti-K.
(2) The reagent should contain anticomplement activity because some complement binding alloantibodies can be detected only by the fact that complement factors are fixed on the red cell membrane as a result of the interaction between red cells and antibody.

(3) The reagent should not agglutinate non-sensitized red cells.

The Group noted that the Working Party on the Standardization of Antiglobulin Reagents has such a reference preparation under trial but that the participants in the trial do not feel that standardization is yet feasible.

It was noted that an alternative approach to standardization is to prepare and standardize separate reagents for anti-IgG and anticomplement (C3). These reagents may then be used as monospecific standards or may be blended to make a polyspecific reference reagent. The composition of the ideal blend, however, has still to be defined. The polyspecific reagent is of greater interest and should have first priority. The Group was informed of some rare examples of auto-antibodies of the IgA class that suggest a possible need for anti-IgA in anti-human globulin sera.

1.5 Red blood cell antigens for immunization

The Group noted the recommendations for immunization of donors to obtain highly potent anti-D plasma for anti-D immunoglobulin. It was desirable to revise these recommendations so as to include the use of frozen red cells for immunization in order to obtain potent plasma for reagents other than anti-A and anti-B. The Group emphasized that the most stringent precautions should be taken to reduce the risk of transmitting hepatitis and of eliciting unwanted antibody responses in these donors.

1.6 Red blood cells for reagent preparation

The Group took note of the need to develop specifications for reagent red blood cells which are used for the quality control testing of blood grouping sera and for testing donor and recipient sera for unexpected antibodies. Although reference materials could be kept frozen in national reference laboratories and by manufacturers, it was clear that this was an area for which WHO should formulate specifications, but international reference preparations would not necessarily be appropriate.

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1.7 Blood group substances A and B

Blood group substances A and B are glycoproteins and glycolipids, which can be obtained from porcine and equine gastric mucosa respectively. They are used to stimulate anti-A antibodies in donors of plasma for ABO blood grouping sera, and in the past they have been used also to neutralize anti-A and anti-B in group O bloods. This practice, however, has been replaced largely by transfusion of group specific blood or by group O red cell concentrates. Although WHO requirements could be written, the Group did not feel there was a great need for either specifications or reference materials at the international level for blood group substances A and B.

2. ANTI-D IMMUNOGLOBULIN

The clinical value of anti-D immunoglobulin in the prevention of Rh immunization is now well established. Excessive dosage of the material is unnecessary and costly, but it is of the utmost importance that an adequate dose be given to prevent Rh0D immunization. Dosage has hitherto been expressed in micrograms of anti-D immunoglobulin determined by isotope labelling methods. The control of preparations is essential, and national specifications have been published.

The Group noted the report 1 of an international collaborative study in which a number of clinical preparations had been assayed against a freeze-dried reference material (coded 68/419). The results of assays by 17 laboratories in 11 countries showed that isotope labelling methods as well as manual and automated haemagglutination techniques gave essentially the same relative potencies when each preparation was assayed against the reference material. The reference material was then calibrated in international units in comparisons by 11 laboratories against the International Standard for Anti-D Blood Typing Serum; it was estimated to contain 150 international units per ampoule. The mean estimate of weight based on 36 direct estimates of anti-D immunoglobulin was 59.2 μg per ampoule.

Preparation 68/419 has already been used widely with a nominal content of 60 μg per ampoule, and the Group agreed that this preparation is valuable in controlling the potency of anti-D immunoglobulin preparations. The Group therefore recommended that WHO take steps to establish this preparation as the International Reference Preparation of Anti-D Immunoglobulin with an assigned unitage of 150 IU per ampoule.

1 Unpublished working document B.G./BLOOD/76.8.
The Group recommended that preparations for clinical use be labelled in international units followed for an interim period by the estimated number of micrograms.

Measurement of anti-D antibodies is carried out when monitoring antibody levels during and after pregnancy and when selecting plasma to be used for the manufacture of anti-D immunoglobulin preparations. Decisions on clinical treatment require accurate estimations, which the Committee agreed should be made against a working standard calibrated in international units.

3. COAGULATION SYSTEMS

3.1 Factor VIII

The first international standard for Factor VIII, which was made in 1966 and established in 1971, was subsequently found to contain hepatitis B surface antigen (HBsAg). Samples of this standard were therefore distributed only after the recipient laboratories were made aware of the hazards associated with its use. Because of this contamination, steps have been taken to replace it with a similar Factor VIII preparation of intermediate purity prepared from plasma shown by radioimmuno-assay to be negative for HBsAg.

An international collaborative assay involving 17 laboratories in 13 countries was organized to calibrate this replacement material in international units. Statistical analysis of the assays so far available has shown that this material is suitable. The Group therefore recommended that the preparation be adopted as the second International Standard for Factor VIII but that its unitage be defined later by the participants in the collaborative assay when analysis of all the results has been completed. Other Factor VIII related activities, such as the Factor VIII-associated antigen, should also be determined in the second international standard so that all clinical measurements may be related to the same material.

It was also recommended that the standard preparations of concentrates of Factor VIII for clinical use should be tested for activated clotting factors, which might give spuriously high estimates of Factor VIII activity.
A national supply of a working standard of plasma and/or concentrate calibrated in international units was recommended for local use in haemophilia centres and laboratories investigating disorders of coagulation.

3.2 Factor IX

The Group was informed that a preparation had been obtained and that an international collaborative assay of the proposed standard had been completed. In this study, two freeze-dried concentrates and one freeze-dried plasma were compared with each other, with fresh normal plasmas, and with local standards. The assays of one preparation of Factor IX (coded 72/32) produced without adding heparin showed good precision with both plasma and concentrate. In the other concentrate the presence of heparin gave rise to non-parallel assays in laboratories testing it at dilutions at which heparin was inhibitory in the assay system. Further study of methods of assaying Factor IX concentrates containing heparin was suggested.

It was recommended that the amounts of clotting factor intermediates in preparation 72/32 should be measured even though, because of the method used in manufacture, they were likely to be small. Several members of the Group volunteered to test preparation 72/32 for these intermediates. Further data collection after the standard has been in use was also recommended to evaluate assay methods for Factor IX.

It was recommended that WHO should, as a matter of urgency, adopt Factor IX concentrate, coded 72/32, as the International Standard with the unitage of 5.62 IU per ampoule.

3.3 Fibrinogen

The Group noted (1) that there are few therapeutic indications for fibrinogen; (2) that, in the rare cases that require fibrinogen therapy, cryoprecipitate should be used instead of a fibrinogen made from large pools of plasma; and (3) that there are adequate national specifications available for the control and characterization of fibrinogen preparations employed for clinical therapy as well as for use as radio-labelled fibrinogen for in vivo diagnosis. Although at the moment a reference preparation of fibrinogen would be of use only for research purposes or for clinical laboratory control, it was agreed that there may soon be a need for an international reference preparation.

BROZOVIC, M. ET AL. Thrombosis and haemostasis (Stuttgart), 35:222 (1976).
3.4 Factors II, VII, X (human) and Factor Xa (bovine)

At present there are no international standards for Factors II, VII, and X and it was agreed that there is a need for international reference materials for these coagulation factors both for the production and control of therapeutic preparations and for the assay of these factors in patients' plasmas. Although the proposed Factor IX standard (coded 72/32) contains Factors II, VII, and X, it was agreed that a similar freeze-dried concentrate should be prepared for an international collaborative assay to compare it with freeze-dried plasma. Such a study is planned in the United Kingdom and the results will be made available to WHO if a suitable reference preparation is identified.

With regard to Factor Xa (bovine), a highly purified preparation has already been made available for research purposes under the auspices of the International Committee for Thrombosis and Haemostasis (ICTH).  

3.5 Antithrombin III

An international standard is needed for antithrombin III for several reasons: (1) highly purified antithrombin III preparations for clinical use are ready for clinical trial; (2) antithrombin III determinations are sometimes needed in patients with deficiencies as well as in women at risk of thrombosis because of oral contraceptive use; (3) the antithrombin III content of Factor IX concentrates may need to be controlled and possibly supplemented; and (4) standardized antithrombin may help resolve some of the problems of heparin standardization.  

It was recommended that the available preparation of highly purified antithrombin III (coded 75/564) should be subjected to an international collaborative assay and that allocation of a unit should be decided on the basis on the results of the study.

Pending the results of the trial, this material (coded 75/564) will be distributed informally to investigators who need it, including the participants in the International Union of Immunological Societies' collaborative assay of serum protein standards for immunological assay of antithrombin III.

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1 Jackson, C. M. et al. Thrombosis and haemostasis (Stuttgart), 35: 479 (1976).

2 Nilsson, I. M. Thrombosis and haemostasis (Stuttgart), 35: 475 (1976); also unpublished working document BLG/BLOOD/76.12.
3.6 Heparin

The biological activities of heparin include the direct inhibition of various steps in the coagulation of blood, inactivation of various clotting components, including kallikrein and plasmin by the acceleration of antithrombin, and stimulation of intravascular lipolysis, as well as the inhibition of complement. Clinically, heparin is used primarily as an anticoagulant (generally by intravenous injection) in thromboembolic disease, in surgery with extracorporeal circulation, and in renal dialysis. Heparin is used also in lower doses (usually by subcutaneous injection) to prevent postoperative thrombosis, and it may be complexed to the surface of intravascular prostheses and equipment to prevent the deposition of thrombi. It is not known, however, what structural features of the molecule are most relevant to these applications or what features produce undesirable characteristics in some preparations, such as the tendency to aggregate platelets.

Different pharmacopoeial procedures, some devised many years ago, may show discrepancies of up to 20% when various heparins are compared with standard preparations. Different methods of preparing assay substrates (blood or plasma), resulting in differing degrees of removal of platelets and use of clotting accelerators, may affect the results of measurement of the relative potencies of different preparations. There are also differences in the reaction between different heparins and the protamines used in heparin neutralization, and these mechanisms are not well understood.

The recent marked rise in the demand for heparin has coincided with a reduction in some established sources of supply. New sources are therefore being explored, and these may yield heparins with other properties. There is also renewed interest in synthetic heparin-like substances. It has become apparent that current heparin preparations are molecularly heterogeneous. Differences in molecular weight may affect both the level obtained in the blood after subcutaneous injection and the time at which the peak occurs. Different methods of fractionating heparins increase the differences in biological effects. The Working Group emphasized the value of identifying in vitro properties that correspond to various clinical effects as well as the need to relate these effects ultimately to chemical structure. It was also suggested that studies should be carried out on heparins made by different methods, which may have special properties, and on those prepared from different tissues.

The need for further work on heparin assay systems was emphasized. It was noted that the results of the United States Pharmacopoeia (USP) assay method had been found to parallel antithrombin activation and that a method using whole rabbit blood had given results similar to those of the British Pharmacopoeia (BP) assay. It was noted also that the rabbit was susceptible to thrombocytopenia induced by some heparins and was therefore useful also in safety testing. In considering the in vivo effects of heparin administration it was suggested that primary differences in biological activity should be distinguished from secondary effects in absorption and elimination.

It was noted that the USP had generously offered WHO a quantity of heparin from the same batch as the current national standard, but it was felt that this should now be used only to study differences between assay methods.

Although not all the characteristics desirable in a heparin standard have yet been defined, the current clinical usage of the substance, such as prophylactic subcutaneous administration, makes the search for new sources of natural heparin and the possible development of synthetic heparins important and the improvement of assays essential. It was noted that standards for antithrombin III and bovine Factor Xa are relevant to the standardization of heparin as is the development of synthetic polypeptide substrates.

4. FIBRINOLYSIS

4.1 Plasmin and plasminogen

The Working Group was informed that a plasmin standard (coded 72/379) had been made available following an international collaborative study and that its use had been recommended by the International Committee for Thrombosis and Haemostasis (ICTH). The standard, which consists of partially purified human plasmin in 50% glycerol, has been shown to be unstable at 20° C or above but highly stable at 4°C or below. Because of the major international need for this standard the Group recommended that WHO adopt 72/379 as an international reference preparation with the unitage of 8.0 IU per ml. The material should be shipped refrigerated with a temperature indicator together

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1 Kirkwood, T. B. et al. *Thrombosis et diathesis haemorrhagica (Stuttgart)*, 34: 20 (1975); also unpublished working documents BLG/BLOOD/76.14 and BLG/BLOOD/76.15a.
with instructions that it should not be used if the temperature had
exceeded 20°C during shipment. In the meantime, efforts should continue
to prepare a pure freeze-dried plasmin with good biological activity
and better stability.

Since variable amounts of plasmin may result from different amounts
and types of plasminogen activators, from different types of plasminogen,
and from differing conditions of plasminogen activation, it was not yet
possible to recommend a single standard plasminogen. Each laboratory
should determine for itself the reagents and conditions needed to obtain
maximum activation for each preparation.

4.2 Urokinase

The Group noted that urokinase is being produced by a number of
manufacturers and is in use as a fibrinolytic activator for the treatment
of thrombosis. Although the heterogeneity of assay results in an inter-
national collaborative study suggested that the assay methods used
were not entirely satisfactory, an international reference preparation
was established in 1968. Since that time, urokinase preparations have
been shown to have two different molecular weight forms (33 000 and
55 000), and it is not yet known whether these forms have different
pharmacological properties or half-life in vivo.¹ These differences in
molecular size were thought to be partly responsible for the variations
in assay results, but newer information indicates that these variations
may be related also to different amounts and types of plasminogen in
the assays used by the laboratories. It is important to confirm whether
assay heterogeneity could be reduced by defining the amount and type
of plasminogen used in the assay in relation to the number of available
active sites in the various preparations of urokinase. The present inter-
national reference preparation could then be further evaluated by an
international collaborative study as a proposed standard.

5. SNAKE VENOMS

5.1 Ancrod

Ancrod, an enzyme purified from the venom of Agkistrodon rhodo-
stoma, is used in patients with intravascular thrombosis to induce con-
trolled defibrination. Although its clinical value has not yet been estab-

¹ Unpublished working documents BLG/BLOOD/76.16 and 16a, and WHO/BS/
66.815.
lished, the accuracy of dosage of this potent thrombin-like enzyme is considered important.

A highly purified material (coded 74/581) prepared and freeze-dried at the National Institute for Biological Standards and Control, London, has been shown to be stable. The Group agreed that an international standard is urgently required and recommended to WHO that action be taken to establish this material as the International Reference Preparation of Ancrod. The manufacturers and the national control laboratory agreed that 55 IU per ampoule should be assigned to it.

5.2 Batroxobin moojeni

Batroxobin moojeni, a purified enzyme prepared from the venom of Bothrops atrox moojeni is another example of a thrombin-like enzyme, and a trial is under way to evaluate its clinical usefulness. The Group noted that a national (British) standard is available and was of the opinion that at the present time WHO should rely on national standards for this type of material. The information that a unit of activity had been assigned to a particular venom should be widely publicized. National authorities and manufacturers should be urged to inform WHO of any national or other units that may have been assigned to these products.

6. STANDARDIZATION IN THE CONTROL OF ORAL ANTICOAGULANT TREATMENT WITH COUMARIN OR INDAJIONE

The Group considered outline proposals2 for the standardization and control of anticoagulant treatment and noted that there were two main aspects: (1) the need for an international reference thromboplastin and other reference thromboplastins; and (2) reference plasmas at the national level, if desired. As far as the origin of the thromboplastins was concerned, the Group noted that there may be objections in some countries to the use of material of human origin. However, a rabbit brain thromboplastin could be used with the sensitivity expressed in terms of the human brain thromboplastin (coded 67/40). The Working Group revised the text of the outline proposals and suggested that

1 Unpublished working document BLG/BLOOD/76.17.
2 Unpublished working document BLG/BLOOD/76.11.
a report on standardization in the control of anticoagulation should be
prepared for consideration by the WHO Expert Committee on Biological
Standardization.¹

6.1 Partial thromboplastin time (cephalin clotting time) reagents

The Group noted that an increasing variety of reagents is available
for the partial thromboplastin time (cephalin clotting time) test and
that several studies on their relative sensitivities are planned or are in
progress. The results, which may indicate the relative value of the
various reference materials, should be considered with a view to the
establishment of international standards for these materials.

7. HUMAN ALBUMIN

The Group noted that the purity and functional properties of human
albumin can now be assessed by several physical and chemical tests ²
and therefore that no international standard is required. Specifications
of human albumin for therapeutic use are adequately covered by national
pharmacopoeias, and specifications for radioactively labelled human
albumin for diagnostic use have been published in at least one national
pharmacopoeia.

8. SPECIFIC IMMUNOGLOBULINS

The Group noted that two categories of human immunoglobulin
preparations are in widespread use for the prevention and treatment
of various infectious diseases:

(1) normal human immunoglobulin, made from pooled human
plasma from a large number of donors (1000 or more); and

(2) specific human immunoglobulin prepared from pooled human
plasma collected from (a) convalescent patients, (b) deliberately immu-
nized donors, or (c) selected antibody-rich plasmas.³

¹ The report on standardization in the control of anticoagulation was considered
and amended by the International Committee for Thrombois and Haemostatis
(ICTH) and the International Committee for Standardization in Hematology (ICSH)
at a meeting in Kyoto, Japan, in September 1976. The amended text is reproduced
as Appendix 2 to this Annex.
² Unpublished working document BLG/BLOOD/76.20.
³ A mixture of (a) and (b) may sometimes be used.
Potency requirements for some of these specific human immunoglobulins have been established to ensure that the preparations contain sufficient antibodies to produce the desired clinical effect. The potency of such preparations must be expressed in international units for quality control, and this also facilitates the exchanging of information regarding their clinical effectiveness.1

8.1 Anti-hepatitis B

Studies have been conducted in several countries to evaluate the clinical effectiveness of anti-hepatitis B immunoglobulin in the prevention of type B hepatitis, but comparison of these studies would have been greatly improved if an international reference material had been available. The Group noted that WHO is arranging an international collaborative study on a batch of freeze-dried human anti-hepatitis B immunoglobulin, in conjunction with a hepatitis B anti-serum of animal origin already available. The Group agreed that WHO should be encouraged to give urgent attention to the provision of a characterized preparation of hepatitis B antibody.

8.2 Anti-varicella zoster

The Group noted that anti-varicella zoster immunoglobulin had proved effective in protecting healthy contacts of acute varicella cases and that clinical studies are currently in progress to evaluate this material in patients exposed to varicella who are immunodeficient because of either immunosuppressive therapy or underlying disease, e.g., acute lymphoblastic leukaemia. A reference material is needed in order to compare the results of these trials in different countries. The Group agreed that a batch of this material should be prepared for an international study and asked the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service to obtain this material. The Group was of the opinion that only plasma from convalescent individuals without malignant diseases should be used in the production of anti-varicella zoster immunoglobulin and that this should be written into the specifications for the product. The international study should be coordinated by WHO and consideration should be given to using the current WHO reference reagent for diagnostic purposes.

1 Unpublished working document BLG/BLOOD/76.21.
8.3 Anti-tetanus, anti-vaccinia, anti-rabies

The Group noted that there are existing international standards for tetanus antitoxin (equine origin), anti-smallpox serum (human origin), and anti-rabies serum (equine origin).\(^1\) International units based on these standards have been used satisfactorily for establishing the potency of national reference preparations for the purpose of quality control. Accordingly, there was no need for additional international reference materials prepared from human immunoglobulin.

With respect to tetanus, however, the Group suggested that consideration be given to a reference preparation of tetanus toxin to be used in the assay of tetanus antitoxin potency when calibrating national standards. The Group recognized that there were some serious technical obstacles to reaching this desirable objective. WHO was urged to organize an international study in order to select the most appropriate preparation.

**PART B:**

**GENERAL CONSIDERATIONS**

1. **BLOOD GROUP NOMENCLATURE — THE NEED FOR A SINGLE INTERNATIONAL SYSTEM FOR Rh**

The Working Group was of the opinion that simplification of Rh nomenclature through the use of the Fisher-Race system was becoming universally accepted. Although Wiener’s great scientific contribution to the description of the Rh system was fully recognized, the Group urged WHO to encourage the use of the Fisher-Race nomenclature in the interests of simplicity and uniformity.

2. **THE NEED FOR REQUIREMENTS FOR THE MANUFACTURE AND CONTROL OF BLOOD PRODUCTS AND THEIR DERIVATIVES**

The Group took note of a draft of “Good practice for the collection and preparation of blood and blood components”. There was much useful information in this draft, but the Group agreed that it could be

\(^1\) Unpublished working document BLG/BLOOD/76.21.
broadened and that recognition should be given to existing international agreements and other appropriate documentation in this field.\(^1\)\(^-\)\(^4\)

The draft was primarily directed to the setting up of a blood transfusion organization, and the Group recognized that several different types of organization might be considered. Their activities might include the recruitment of donors, blood collection, processing, component preparation, distribution, compatibility testing, administration of blood or its components and derivatives to patients, the production of plasma derivatives, and the provision of laboratory reagents and of certain types of equipment such as blood containers complete with preservative solutions.

The Group agreed that there is an urgent need for WHO to formulate requirements for the production and quality control of blood products and that it is essential to include consideration of donors and source materials. It also stressed the need for overall guidance on the organization of transfusion services, including appropriate technical specifications.

Since the various requirements for the control of blood and related substances must take into consideration not only the activities carried out in a blood transfusion service but the operations of fractionation and control, it is essential that they be integrated. In order to achieve this most efficiently, the Group urged that WHO take a united approach to these requirements.

**PART C:**

**COLLABORATION WITH INTERNATIONAL SCIENTIFIC ORGANIZATIONS**

Over the past decade disciplines in biology have become more specialized and technology more refined and international societies are now dealing in depth with individual subjects. The scientists able to give advice on the most suitable biological standards for these disci-

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4 Meeting on the utilization and supply of human blood and blood products, Berne, December 1975. Unpublished WHO document LAB.76.2. This document is available on request from Health Laboratory Technology, World Health Organization, 1211 Geneva 27, Switzerland.
plines (endocrinology, haematology, immunology, microbiology, etc.) are members of such scientific societies and many of them are now organized into working groups to satisfy these needs.

It is entirely appropriate, therefore, for WHO to establish close links with these societies, not only to receive a consensus on suitable standards but also to keep abreast of work that may be in progress. Frequently, the work of such societies has led to the establishment of standards with the ultimate objective of submitting them to WHO for approval as international standards.

The meeting was informed of WHO’s administrative mechanism for establishing biological international standards, reference preparations, and reference reagents. The usual procedure is to present a report through the WHO Secretariat to the WHO Expert Committee on Biological Standardization, which is responsible for the establishment of these preparations. Another mechanism in accordance with resolution WHA26.32 (part II, paragraph 1) is to circulate the data to members of WHO’s Expert Advisory Panel on Biological Standardization and to other experts. In any case a report should be submitted containing information on the need for that particular standard, the selection and handling of the materials, their characterization, the design and results of the collaborative study, and the analysis and interpretation of these results, together with recommendations of a unitage as agreed by the participants. After the scientific and administrative evaluation of any comments received, the Director-General may establish the preparation. The need for attention to detail is paramount; the criteria for the containers, the stability of the international biological preparation, and the validity of the design of the collaborative study and of the appropriate statistical analyses must be well established.

Many of the preparations are expensive and their characterization often requires a great deal of time. In order to ensure that the preparations meet with the approval of the Expert Committee on Biological Standardization, the Group recommended the formulation of guidelines for the establishment of standards. Such guidelines should be widely distributed to scientific societies for their consideration and might also be useful to national control authorities. Notes on the preparation of materials to serve as international biological standards, reference preparations, and reference reagents 1 are available, but they should be brought up to date and coupled with guidelines for the design for collaborative assays and notes on the presentation of data.

1 Unpublished working document WHO/BS/773.65.
PART D:
RECOMMENDATIONS OF THE WORKING GROUP

The Working Group recommended WHO:

(1) To urge national authorities to express the potencies of blood grouping sera in international units where these are available instead of using titres (Part A, section 1.1).

(2) To establish the material coded 67/160 as the International Standard for Anti-c Incomplete Blood Typing Serum and to assign a unitage of 64 IU per ampoule to it (Part A, section 1.2).

(3) To formulate general requirements for the production and testing of blood grouping antisera and other ancillary reagents (Part A, section 1.3).

(4) To revise the recommendations for immunization of donors to obtain highly potent anti-D plasma for anti-D immunoglobulin (Part A, section 1.5) and to formulate specifications for reagent red blood cells used for the quality control testing of blood grouping sera (Part A, section 1.6).

(5) To establish the material coded 68/419 as the International Reference Preparation of Anti-D Immunoglobulin and to assign a unitage of 150 IU per ampoule to it (Part A, section 2).

(6) To establish the material coded 72/32 as the International Standard for Factor IX concentrate and to assign a unitage of 5.62 IU per ampoule to it (Part A, section 3.2).

(7) To establish the material coded 72/379 as the International Reference Preparation of Plasmin and to assign a unitage of 8.0 IU per ml to it (Part A, section 4.1).

(8) To establish the material coded 74/581 as the International Reference Preparation of Ancrod and to assign a unitage of 55 IU per ampoule to it (Part A, section 5.1).

(9) To seek information from all national authorities on the units that may have been assigned to the activity of snake venoms used for the control of defibrination (Part A, section 5.2).

(10) To consider the action suggested for standardization in the control of anticoagulation (Appendix 2) modified if necessary in the light of comments by the ICTH and ICSH (Part A, section 6).
(11) To give urgent attention to the provision of a characterized preparation of hepatitis B antibody (Part A, section 8.1).

(12) To arrange for a collaborative study of the assay of anti-varicella zoster immunoglobulin and of the possibility of using the WHO reference reagent for diagnostic purposes (Part A, section 8.2).

(13) To arrange a collaborative study for the selection of a suitable reference preparation of tetanus toxin for use in the assay of potency of tetanus antitoxin (Part A, section 8.3).

(14) To encourage the use of the Fisher-Race nomenclature for the Rh blood group system (Part B, section 1).

(15) To formulate comprehensive requirements for the production and quality control of blood products and to include guidance on the organization of transfusion services and the appropriate technical specifications (Part B, section 2).

(16) To formulate guidelines on the procurement, characterization, and calibration of international standards, international reference preparations, and international reference reagents (Part C).

Appendix 1

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STANDARDIZATION IN THE CONTROL OF ANTICOAGULATION (ORAL)

Outline Proposals

This report outlines proposals for national and international standardization of reagents used in the laboratory control of anticoagulant treatment with coumarin drugs. Reference thromboplastins are proposed at the international and national levels. Reference plasmas are also proposed at the national level for calibration of thromboplastins, for quality control, and as independent reference preparations.

1. Anticoagulation and the need for standardization

Treatment and prophylaxis of thrombotic disorders with coumarin (and sometimes indandione) oral anticoagulant drugs are widely practised. These compounds act by reducing the clotting activities of Factors II, VII, IX, and X in the blood by interfering with their synthesis in the liver. The dose of these powerful drugs must be adjusted to ensure that adequate but not excessive anticoagulation is achieved. This adjustment is initially frequent and must continue routinely for months or years as long as these drugs are taken. It is usually made according to the results of the prothrombin time test (or a derived procedure) on the patient’s blood. The test involves the use of a tissue extract called thromboplastin to confine the sequence of reactions leading to coagulation mainly to those factors reduced in the blood by coumarin treatment—the sequence known as the “extrinsic pathway”.

Various types of thromboplastin are made commercially. Thromboplastins vary in their reaction to the clotting defets produced by coumarin. They may differ (1) in the degree to which they are inhibited by the molecular variants of the clotting factors synthesized under coumarin treatment, which are known as PIVKAs—Proteins Induced by Vitamin K Antagonist(s)—(2) in their dependence on Factor V in the tested plasma, and (3) in their reaction with clotting factors activated by surface contact. Thus, different thromboplastins give widely different clotting times on the same sample of coumarin plasma.

Standardization is therefore essential for proper monitoring of treatment. Standardization of prothrombin time tests has been extensively studied and frequently reviewed but has not yet been achieved. Attempts at standardization have followed three main approaches: the central provision of a single type of thromboplastin; the use of stable reference preparations of the main types of thromboplastins; and the use of reference plasmas.

It has been found possible to relate the clotting time (prothrombin time) obtained with one thromboplastin to that using another by expressing the clotting times as ratios (see section 4 below). International studies have confirmed that the activities of freeze-dried reference thromboplastins can be reproducibly related to each other in different laboratories in this way, and a number of widely used thromboplastins

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1 Prepared by the WHO Working Group on the Standardization of Human Blood Products and Related Substances, 5-10 July 1976, and amended according to comments made at the meeting of the International Committee for Thrombosis and Haemostasis (ICTH) and the International Committee for Standardization in Haematology (ICSH) at Kyoto, Japan, in September 1976.
have been related to a single reference preparation. The stability of various clotting factors in freeze-dried coumarin plasmas has been assessed, and it has been shown that artificially depleted plasmas can be reproducibly manufactured.

2. Aim
The aim of these outline proposals is to establish a system for international standardization in the control of oral anticoagulant treatment. Any proposed system must enable prothrombin times obtained with thromboplastins of various types to be related to a common scale so that anticoagulant effects measured in samples of patients' plasma are comparable, irrespective of which thromboplastin is used.

3. Definitions
The following definitions are proposed for the purposes of the present appendix only.

Prothrombin time. The clotting time of a plasma sample in the presence of a preparation of thromboplastin.¹

Thromboplastin. A tissue extract with the ability to accelerate the activation of blood coagulation through the extrinsic pathway, thus bypassing some of the reactions of the intrinsic pathway. Thromboplastins prepared from mammalian tissues contain proteins and phospholipids. A preparation of a thromboplastin consisting of a tissue extract alone is termed "plain"; when the preparation has additional components such as fibrinogen, Factor V, or calcium, it is termed "combined". Thromboplastins may also be grouped into types, each made from different tissue sources, e.g., human, bovine, rabbit brain or lung, or human placenta.

Reagent plasmas. Plasmas taken with suitable precautions as described in the prothrombin time method.² Reagent plasmas comprise the following types:

(a) normal plasma: plasma obtained from a healthy person.
(b) coumarin plasma: plasma obtained from a patient stabilized on oral anticoagulant treatment for six weeks or more, but with no other interfering haemostatic defect.
(c) artificial plasmas: normal plasmas treated in various ways in vitro with the intention of simulating the effects of oral anticoagulant treatment.
(d) fresh plasma: plasma used in the prothrombin time test within a few hours of collection.
(e) stored plasma: plasma suitably frozen within a few hours of collection or preparation. It may then be freeze-dried.

Reference plasma. A batch of stored reagent plasma, established by a national control authority.

National reference laboratory. A laboratory having nationally recognized expertise, designated by the national authority. The laboratory should have access to fresh normal and coumarin plasmas.

4. Calibration of one thromboplastin against another
The calibration of one thromboplastin against another involves determining the relationship between the results obtained with two thromboplastins in parallel tests on normal and coumarin plasmas.

In the calibration procedure originally described, prothrombin times were determined in parallel with the test and reference thromboplastins on a number of reagent normal plasmas and a much larger number of reagent coumarin plasmas.

When the ratios of coumarin plasma clotting time to mean normal clotting time for each coumarin plasma obtained with each of the two thromboplastins concerned are plotted against each other the relationship is a straight line. This provides the basis for calibrating one thromboplastin in terms of the other. The calibration line is most simply derived by drawing a straight line from the point (1,1) through the point representing the mean ratio obtained with each thromboplastin.

**Calibration constant**

A plasma sample from a coumarin-treated patient tested with a given batch of thromboplastin gives a prothrombin time expressed in seconds. When a normal plasma is tested with the same thromboplastin a different time is obtained. The ratio of the two clotting times (patient’s plasma/normal plasma) may be derived from the mean of several such measurements. The same process may be carried out using the proposed International Reference Preparation of Thromboplastin (Human, Combined), giving another ratio. The two ratios may then be plotted as in Fig. 1, giving point A. If the process is repeated for various coumarin plasmas, the plotted ratios will be found to lie approximately on a straight line passing through the point (1,1). The slope of this line is called the calibration constant of the thromboplastin. In practice there is no need to derive more than one set of ratios because a straight line can then be immediately drawn through the plotted point and the point (1,1). Conversely, if the calibration constant is known the calibration line can easily be drawn since it must pass through the point (1,1) with a slope equal to the calibration constant. Since all thromboplastins are ultimately calibrated against the proposed International Reference Preparation of Thromboplastin (Human, Combined), the calibration constant of this preparation is defined as 1.0.

![Fig. 1](image-url)

Calibration constant of a thromboplastin
However, supplies of the proposed International Reference Preparation of Thromboplastin (Human, Combined) are limited, and it is necessary to follow a less direct approach to calibration, using another international reference thromboplastin such as the International Reference Preparation of Thromboplastin (Rabbit, Plain). This latter preparation can be calibrated against the former yielding a calibration constant of 0.60. This exercise would be carried out in a national reference laboratory, and the need for it would be infrequent. Any given batch of thromboplastin can then be calibrated against the International Reference Preparation of Thromboplastin (Rabbit, Plain). If the slope obtained is 0.65, say, the true calibration constant of the batch will be 0.65 x 0.60 = 0.39.

**International calibrated ratio**

The ratio of the clotting time for a patient’s plasma to that for a normal plasma is generally determined using a given batch of calibrated working thromboplastin. The ratio obtained is then converted to the value that would have been obtained had the measurement been carried out using the International Reference Preparation of Thromboplastin (Human, Combined). This may be done graphically by drawing a straight-line graph, as shown in Fig. 2, passing through the point (1,1) and having a slope equal to the calibration constant of the working thromboplastin. The international calibrated ratio may then be read off as indicated. Alternatively, the conversion may be effected by using the simple formula:

\[
\text{ratio on calibrated scale} = 1 + \left[ \frac{(r-1)}{k} \right]
\]

where \( r \) is the ratio using the given thromboplastin and \( k \) is the calibration constant of that thromboplastin.

![Fig. 2](image)

**Fig. 2**

Degree of anticoagulation defect of a patient’s plasma on the international calibrated scale

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The international calibrated ratio is a value for the plasma sample independent of the thromboplastin used. It is then for the clinician to decide, on the basis of the international calibrated ratio determined for his patient, whether to adjust the dose of coumarin.

5. The basis of standardization

The proposed measures to effect standardization are based on the following assertions.

1. Different types of thromboplastin can be used for assessment of the coumarin defect in plasma.

2. There are many manufacturers of preparations of thromboplastin, and many batches of different types of thromboplastin are produced each year.

3. Thromboplastin preparations may be calibrated against each other using the ratio method. To determine the slope of the calibration line with sufficient precision it has been customary to test five normal plasmas and 20 coumarin plasmas per day, for five days.

4. The definition of the calibration constant of a thromboplastin is given in section 4.

5. Calibration is more precise (i.e., the standard deviation of the slope of the line is less) when a preparation of thromboplastin is calibrated against a reference thromboplastin of the same type, rather than against one of a different type.

6. The establishment of international reference preparations of thromboplastins of the main types in use would enable any ratio determined with those types of thromboplastin to be reported on a single common scale.

7. Reference preparations of thromboplastins are found to be stable when freeze-dried and sealed in ampoules according to the procedure used to prepare international biological standards.1

8. In some countries it may be unrealistic to expect to obtain individual fresh samples of all the coumarin plasmas required for the calibration of every batch of thromboplastin of every type used or made within the country.

9. Coumarin plasmas can be grouped into those with short, medium, and long clotting times. When pooled, these plasmas are stable for calibration purposes for up to a year if suitably frozen and stored below −80°C or if suitably freeze-dried, sealed in ampoules, and stored at −20°C.

10. In practice, such plasmas can be used in place of fresh plasmas for calibrating one thromboplastin against another of the same type.

11. A fundamentally different approach to standardization is to establish reference plasmas assayed for the levels of those clotting factors that are reduced by coumarin treatment. Reference plasmas would be chosen with levels of clotting factors corresponding to given degrees of anticoagulation.

12. Manufacturers of thromboplastins would determine the mean prothrombin time for each batch of thromboplastin using each plasma.

13. Evidence obtained from comparisons of the behaviour of patients' fresh plasmas and freeze-dried reference plasmas with pairs of thromboplastins suggests that coumarin reagent plasmas would most likely be useful in such a scheme; but for a group of thromboplastins of the same type, artificial reagent plasmas might also prove satisfactory.

(14) The international calibrated scale could be linked to a reference plasma scheme by determining the international calibrated ratios of the reference plasmas.

6. Proposals for standardization

The following measures to effect international and national standardization of anticoagulant treatment are proposed.

6.1 At the international level

International reference preparations of the main types of thromboplastins would be established. Calibration constants relative to preparation 67/40 have already been determined in past studies and confirmed in the recent international ICSH/ICTH collaborative study. They are as follows:

(a) Thromboplastin, Bovine, Combined (coded 68/434): calibration constant = 1.0.
(b) Thromboplastin, Human, Plain (coded 69/223): calibration constant = 0.90.
(c) Thromboplastin, Rabbit, Plain (coded 70/178): calibration constant = 0.60.

A reference preparation should be established for each additional type of thromboplastin that becomes widely used and that cannot be readily calibrated using existing reference preparations.

6.2 At the national level

It would be the manufacturers' responsibility to calibrate and label each batch of thromboplastin with its calibration constant or with the prothrombin times obtained with the agreed reference plasmas, or both. It would be the responsibility of the national control authority, through its national reference laboratory, to effect appropriate control of manufacture, testing, and labelling.

Two possible schemes of standardization are envisaged—the reference thromboplastin scheme and the reference plasma scheme—but linkage between the two might be achieved.

Reference thromboplastin scheme. The national reference laboratory would establish national reference preparations for each type of thromboplastin in use in that country. These would be calibrated nationally using the appropriate international reference preparations and labelled with their calibration constants. At this stage it would be important to take account of bias due to different methods of endpoint determination. If national calibration was undertaken with fresh normal and coumarin plasmas, the work could be done only at (or in association with) clinical centres having access to these materials. If sufficient quantities of coumarin reagent plasmas were made available, manufacturers could undertake this calibration.

Reference plasma scheme. Manufacturers would determine with each batch of their thromboplastin the prothrombin time obtained with each of the reference plasmas. Each batch of thromboplastin would be labelled with the prothrombin time for each specified reference plasma.

Linking the reference thromboplastin and reference plasma schemes. If the reference plasmas were themselves labelled with their international calibrated ratios the two schemes could be linked.

6.3 Local quality control

An essential part of standardization is local quality control. One aspect is to confirm that the same reagents give comparable results when used at different times and in different places. This could be done by distributing batches of coded materials
against which local reagents could be tested. Reagent plasmas have an obvious role as materials for distribution; artificial plasmas have proved useful at several centres in quality control schemes.

7. Recommendations

It is recommended:

(1) that the proposed international reference preparation of thromboplastin (human, combined) (coded 67/40) be established as the International Reference Preparation with a defined calibration constant of 1.0.

(2) that the proposed international reference preparation of thromboplastin (bovine) (coded 68/434), thromboplastin (human, plain) (coded 69/223), and thromboplastin (rabbit, plain) (coded 70/178) be established as International Reference Preparations with calibration constants assigned in relation to 67/40.

(3) that, before a reference plasma scheme is adopted, criteria for the choice of reference plasmas should be agreed.

(4) that WHO publish guidelines for national authorities on how standardization can be effected at the national level, following the outlines contained in this paper.

(5) that national standardization be effected by establishing national reference thromboplastins calibrated in terms of the appropriate International Reference Preparation. The approach to standardization adopted in a given country will depend on local circumstances such as previous local experience of reference thromboplastins or plasmas, but, whichever approach is chosen, national reference thromboplastins will provide a basis for using a common international scale.
Annex 2

REQUIREMENTS FOR MENINGOCOCCAL POLYSACCHARIDE VACCINE
(Requirements for Biological Substances No. 23)

Addendum 1976

The WHO Expert Committee on Biological Standardization at its twenty-seventh meeting adopted Requirements for Meningococcal Polysaccharide Vaccine which were published in its report.² During the discussion it was reported that at that time (December 1975) the requirement for the value of the distribution constant \( K_D \) was undergoing rapid change as the technology developed. Nevertheless, in view of the extensive use of the vaccine, it was considered necessary to include a specification for molecular size. During the past year much more experience has been gained in the determination of molecular size, and it is proposed that Part A, section 3.4.6, be replaced by the following:

"3.4.6 Molecular size

The molecular size of each lot of purified polysaccharide shall be estimated by gel filtration using Sepharose 4B. Chromatography shall be carried out in a solvent having an ionic strength of 0.2 mol/kg. The molecular size shall be determined by measuring the recovery of the polysaccharide eluted before a \( K_D \) of 0.50 is reached. At least 65% of the Group A polysaccharide and at least 75% of the Group C polysaccharide shall be recovered from the column before a \( K_D \) value of 0.50 is reached."

Footnote 2 is deleted.

Part A, section 5.6, should be replaced by the following:

"5.6 Estimation of molecular size

The molecular size of the polysaccharide in at least one final container from each filling lot shall be determined by Sepharose 4B gel filtration as outlined in Part A, section 3.4.6. At least 65% of the Group A polysaccharide and at least 75% of the Group C polysaccharide shall be recovered in the column effluent before a \( K_D \) value of 0.50 is reached."

Footnote 1 is deleted.

The purification procedures of the polysaccharide have improved to such an extent that it is now possible to inoculate 10 times the quantities into a rabbit for the pyrogenicity test than was previously possible without inducing a rise in temperature. The first paragraph of Part A, section 5.5.1, should therefore read as follows:

"Each filling lot shall be tested for pyrogenicity by intravenous injection into rabbits. Three or more healthy rabbits that have not previously received injections shall be used. The vaccine, reconstituted in the form in which it is to be used, shall be diluted further in pyrogen-free physiological saline so that each rabbit shall receive, by injection into the ear vein, the following doses of dry weight polysaccharide per kilogram of rabbit weight:

- Group A vaccine, 0.025 µg
- Group C vaccine, 0.025 µg
- combined Groups A and C vaccine, 0.050 µg"

In the Appendix to the Requirements, section 3, subsection (4), the last sentence of paragraph (d) should be deleted, together with footnote 1.
INTRODUCTION

Rubella (German measles) gives rise to a mild exanthematosus illness, accompanied by few constitutional symptoms, and occurs most commonly in childhood. If the infection occurs in a woman in early pregnancy, however, the virus may cross the placenta to reach the fetus, in which the infection can induce birth defects. These defects may be serious and permanent and include congenital heart disease, cataract formation, deafness, and mental retardation. The prevention of fetal infection, therefore, is the primary purpose of rubella immunization.

1 Prepared by the following members of the WHO Secretariat: Dr A. J. Beals, The Wellcome Research Laboratories, Beckenham, Kent, England (Consultant); Dr C. Huegelen, Director of Research, Therapeutic Research and Industry, Genval, Belgium (Consultant); Dr P. D. Parkman, Deputy Director, Bureau of Biologicals, Food and Drug Administration, Bethesda, MD, USA (Consultant); and Dr F. T. Perkins, Chief, Biologicals, WHO, Geneva, Switzerland.
The WHO Expert Committee on Biological Standardization in 1965 recognized the need for an international standard for anti-rubella serum for use in the assay of rubella antibodies and in the control of specific (anti-rubella) immunoglobulins. In 1966 the nineteenth Expert Committee on Biological Standardization established the first International Reference Preparation for Anti-Rubella Serum and in 1970 the twenty-third Expert Committee established a more suitable preparation as the second International Reference Preparation for Anti-Rubella Serum.

Since there are several strains of virus and at least four different cell substrates used for their growth it was not considered appropriate to select one of them for the control of all strains. Therefore the responsibility for the provision of a virus preparation used in the control of virus titre should be undertaken by the national control authority, and this has been referred to in Part A, section 1.3 and Part B, section 1 of the present requirements.

The following international requirements for rubella vaccines have been fitted into the framework adopted in the Requirements for Biological Substances Nos. 1–23 already published by WHO and in drafting them account has been taken of the opinions of consultants, the regulations and requirements for the manufacture and control of rubella vaccines that have been formulated in a number of countries, and information from both published and unpublished reports. In addition, opinions and data have been received from a number of experts whose assistance is gratefully acknowledged below.

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GENERAL CONSIDERATIONS

At the present time (1976) over 50 million people throughout the world have been vaccinated with live rubella virus vaccines. Such vaccines have been shown to be safe and effective in studies conducted prior to their approval by national control authorities and through the extensive experience gained in actual clinical use.

Three virus strains are used widely for vaccine production, these are: the HPV-77 virus strain, originally recovered from a young adult with typical rubella, the Cendehill virus strain isolated from the urine of a child with rubella, and the RA-27/3 virus strain recovered from the explanted tissues of a fetus obtained at therapeutic abortion because of maternal rubella virus infection. These viruses were attenuated by repeated serial passage in cell cultures and they are currently produced in duck embryo cells (HPV-77 DES), rabbit kidney cells (Cendehill), and human diploid cells (RA-27/3). All three vaccines are administered by
the subcutaneous route. The vaccine viruses produce infection associated with minimal amounts of pharyngeal virus shedding and an immune response in approximately 95% of recipients. These infections differ from those produced by the wild virus in the limited duration and degree of virus excreted by the pharynx, the low incidence of detectable viraemia, and, as with other attenuated vaccines, a lower titre of antibody response.

In addition to these three strains the Takahashi and Matsuura attenuated strains, grown in rabbit or primary Japanese quail embryo fibroblasts, have been used on a large scale in Japan.

Although communicability of the vaccine-induced infections was considered as a possibility, considerable evidence from programmes of mass vaccination has shown that this does not occur. The clinical consequences of the natural disease (e.g., fever, rash, lymphadenopathy) are of uncommon occurrence after vaccination. Transient joint and peripheral nervous system involvement manifested clinically as arthralgia or arthritis as well as paraesthesia of the extremities which occur commonly in the natural disease may also be seen in vaccinees. Such symptoms are related to age and sex and have been noted most commonly in adult females. The rates observed vary widely depending on the method used for observation and follow up. In practice the rate of such reaction is usually less than 1% in children and less than 10% in adults. The immunity produced appears to be long-lasting, and antibodies have been shown to persist for at least seven years. It is anticipated that long-term protection will ensue but continued observations of vaccinees will be necessary to determine the exact duration of the protective effect.

Pregnant women should not receive rubella virus vaccine. Although to date no case of congenital defect has been attributed to the vaccine viruses, the attenuated viruses have crossed the placenta and have been recovered from the fetus. Thus, while the evidence suggests a lesser degree of risk than with the natural virus, no assurance of safety can be given to the pregnant woman. Pregnancy or the likelihood of pregnancy during the period from two months before until two months after vaccination is an absolute contraindication to vaccination.

The extensive use of rubella vaccine has produced a significant decline in the number of reported cases of both rubella and the congenital rubella syndrome. With this evidence of efficacy, the use of attenuated vaccines is becoming increasingly common throughout the world.

1 In addition to administration of the vaccine by the subcutaneous route, studies are in progress with the administration of the RA27/3 strain by the intranasal route.
For this reason there is a need to develop international requirements for the production and testing of live rubella vaccine.

The technical considerations relating to the production of these vaccines in certain particulars embody features reflecting scientific and technical advances during the past decade, which have resulted in considerable progress in the avoidance of difficulties associated with the introduction of extraneous agents from the cell substrates themselves. They are (1) the use of a closed colony of animals that are continuously monitored for known pathogenic agents and used to provide tissues for producing the cell substrate, and (2) the use of serially propagated human cell strains fully characterized with respect to karyology, lack of tumourigenicity (as measured by lack of heterotransplantability), and freedom from extraneous agents. Experience over the past seven years using these types of cell substrates has shown no contamination intrinsic to the cell batches used. These advances are embodied in the present requirements. They place safety testing on a logical basis and concentrate the testing for extraneous agents on samples taken at the stages of the production process most likely to give maximum assurance of the safety of the final product. Several specific points in this connection are worthy of mention. First, it is required that each isolated colony or flock be continuously monitored for the presence of pathogenic microorganisms and similarly it is also required that the diploid cell bank be fully tested for the presence of contaminants and the maintenance of normal karyology. These procedures provide an important initial step that introduces a much higher degree of safety to the entire process of cell substrate production. Second, the emphasis of the safety tests on the control cells has been placed late in the production cycle in order to provide the maximum opportunity for the detection of any extraneous contamination of the cell substrate. Third, since serum has been found to be a potential source of introduction of extraneous agents into vaccines, a requirement for the testing of bovine serum used in the cell culture medium is included.

Certain differences in these requirements from some national requirements will be noted. For example, changes have been introduced in the animal and cell culture testing procedures included in the requirements of certain national control authorities as well as in WHO requirements for the control of vaccines produced in cells from sources that have not been so carefully monitored.

These regulations do not include marker tests because there is no definitive laboratory test for attenuation of rubella virus analogous to the neurovirulence tests for poliomyelitis virus vaccine (oral) and
yellow fever virus vaccines. However, a test for identity of the attenuated
strain is included. Experience has shown that the maintenance of a
standard operating procedure, including a standard temperature of
incubation and the use of a seed lot system, results in a consistent product
maintaining a regular pattern of attenuation and immunogenicity.
Nevertheless studies should continue in a search for suitable marker tests.

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 require-
ments. The parts of each section that are printed in small type are
comments and recommendations for guidance. In order to facilitate
the international distribution of vaccine made in accordance with these
requirements a summary protocol for the recording of the results of the
tests is included as an appendix.

Should individual countries wish to adopt these requirements as the
basis of their national regulations concerning rubella vaccine, it is
recommended that a clause be included permitting modifications of
manufacturing requirements on the condition that it be demonstrated,
to the satisfaction of the national control authority, that such modified
requirements ensure a degree of safety and a potency of the vaccine at
least equal to those provided by the requirements formulated below.
It is desirable that 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 rubella vivum*. The proper
name shall be the equivalent of the international name in the language
of the country of origin.

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

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1.2 Descriptive definition

_Vaccinum rubella vivum_ is a preparation of live attenuated rubella virus grown in _in vitro_ cultures of suitable cells. The preparation shall satisfy all the requirements formulated below.

At present, live rubella vaccine is stabilized by lyophilization and is available for distribution only in that form.

1.3 International reference preparation and international unit

Since no international standards or reference preparations of live rubella vaccine have yet been established, no requirements for potency based on such standards or preparations can be formulated. National control authorities should provide a reference preparation of live rubella virus for use in tests of virus concentration (see Part A, section 3.5.3).

The second International Reference Preparation of Anti-Rubella Serum (established in 1970) is dispensed in ampoules containing 145.95 mg of freeze-dried human normal immunoglobulin. The International Unit is defined as the activity contained in 0.14595 mg of the second International Reference Preparation. Each ampoule, therefore, contains 1000 International Units.

The International Reference Preparation of Anti-Rubella Serum is in the custody of the International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen. Samples are distributed, free of charge, on request to national control laboratories. The International Reference Preparation is intended for the calibration of national standards and reference preparations for use in the manufacture and laboratory control of anti-rubella serum and of hyperimmunoglobulin.

1.4 Terminology

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

_Original vaccine_. A monovalent vaccine, prepared from the original seed virus, and shown on administration to man in extensive field trials to be immunogenic and free from harmful effects.

_Seed lot_. A quantity of virus derived from an original vaccine processed together and of uniform composition. Seed lots are not more passages removed from the original seed virus than a number approved by the national control authority.

_Cell seed_. A quantity of cells derived from a single human tissue and of uniform composition, stored frozen at −70°C or below in aliquots, one of which would be used for the production of a single harvest.
**Single harvest.** A virus suspension harvested from cell cultures prepared from tissues that have been processed together. For vaccine made in human diploid cells the single harvest shall be made from the cells prepared from one ampoule of cell seed bank.

**Bulk suspension.** A pool of a number of single harvests.

**Final bulk.** The finished biological preparation present in the container from which the final containers are filled. The final bulk may be prepared from one clarified bulk suspension, or from a blend of clarified bulk suspensions, or from a dilution thereof.

**Filling lot (final lot).** A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling or preparation of the finished vaccine. A filling lot must, therefore, consist of finished material filled in one working session and dried together.

**Tissue culture infective dose 50% (TCID50).** The quantity of a virus suspension that will infect 50% of cell cultures.

**Plaque-forming unit (PFU).** The smallest quantity of a virus suspension that will produce a plaque in monolayer cell cultures.

### 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)¹ shall apply to establishments manufacturing rubella vaccine, with the addition of the following requirements.

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

The production of rubella 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 areas are immune to rubella.

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

Visitors and persons not directly concerned with the production processes shall not be permitted to enter the production area.

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

3.1 Control of source materials

3.1.1 Virus strains

The strain of rubella virus used in the production of rubella vaccine shall be identified by historical records, which shall include information on the origin of the strain as well as on the method used in the attenuation of it. The strain shall be shown to be immunogenic and only strains that are approved by the national control authority shall be used.

3.1.2 Cell cultures for virus propagation

Rubella virus used in the production of rubella vaccine shall be propagated in cell cultures approved by the national control authority.

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 rabbit kidney cell cultures are used for the propagation of rubella virus, the rabbits (Oryctolagus cuniculus) from which the kidneys are taken shall be from a closed colony continuously monitored for the ab-
sence of coccidiosis, myxomatosis, rabbit pox, fibromatosis, herpesvirus cuniculi, *Mycobacterium tuberculosis*, *Nosema cuniculi*, *Toxoplasma gondii*, rabbit kidney vacuolating virus, syncytium virus of rabbits, and other pathogenic microorganisms and viral agents naturally occurring in rabbits.

If duck (*Anas pekin*) embryo fibroblast cultures are used for the propagation of rubella virus, the eggs used as the source of cells shall be derived from a closed flock continuously monitored for the absence of salmonella infections, *Mycobacterium tuberculosis* (avian), fowl pox, Rous sarcoma virus, avian leucosis viruses, duck hepatitis, duck plague, Newcastle disease, duck influenza, arboviruses, ornithosis, Marek's disease virus, mycoplasma, and other pathogenic microorganisms and viral agents naturally occurring in ducks.

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

In some countries biochemical tests for the detection of RNA 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.

If human diploid cells are used for the propagation of rubella 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. The supernatant fluids shall also have been shown by tests in cell cultures to be free from adventitious agents. The cells shall have been shown to be diploid and stable with respect to karyology and morphology and to meet the requirements given in the subsections of Part A, section 3.2, throughout their finite life-span. Each manufacturer shall show to the satisfaction of the national control authority that the cell substrate laid down as a bank from each initial culture received conforms with the tests outlined in this section for freedom from extraneous agents in animals and eggs, for lack of heterotransplantability, and for normal karyology throughout its normal life-span.

In some countries the cells are examined also by ultra-thin sections and by negative staining under the electron microscope.
The tests in animals and eggs for adventitious agents shall include one of inoculating each of the following groups of animals with the cells under test by the intramuscular route, using at least $10^6$ cells for each group:

- 2 litters of suckling mice, totalling at least 10 animals, less than 24 hours old;
- 10 adult mice;
- 5 guineapigs; and
- 5 rabbits.

Cells shall also be injected into the allantoic cavity 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 guineapig and chick or other avian red cells for the presence of haemagglutinins.

In some countries the suckling and adult mice are also inoculated by the intracerebral route.

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

Suitable tests using immunosuppressed animals may be made as follows. Some $10^6$ cells obtained from cultures at the same passage level as those used for vaccine production are injected into either (a) newborn mice or hamsters treated with antilymphocytic serum, (b) athymic mice (made nu/nu genotype), or (c) thymectomized mice irradiated and reconstituted (T−B+).

Some of the same group of animals should be inoculated with a similar dose of MeLa 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.

The cells are suitable for vaccine production if at least 80% of the animals inoculated with the cells remain healthy and survive the observation period, none of the animals or eggs shows evidence of the presence in the cell cultures of any adventitious agent, and none of the animals shows evidence of tumour formation from the cells.
The cell seed shall also have been shown to yield cell cultures capable of producing vaccine that has been found to be safe and antigenic in man.

3.1.2.1 Serum used in cell culture medium. Serum used for the propagation of cells for rubella vaccine production 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)\(^1\) as well as freedom from mycoplasma and bovine viruses by methods approved by the national control authority. It shall also be shown to be free from inhibitors to rubella virus.

In some countries sera are examined for freedom from phage.

3.1.3 Seed lot system

The production of vaccine shall be based on the seed lot system.

Each seed lot shall be identified as rubella virus by appropriate methods (see Part A, section 3.5.3).

It is recommended that a large seed lot be set aside as the basic material to which the manufacturer can return for the preparation of batches of vaccine.

A seed lot shall be stored in a separate refrigerator at a temperature lower than \(-20\)\(^\circ\)C or, if not lyophilized, at a temperature of \(-60\)\(^\circ\)C or lower.

All vaccine lots shall contain rubella virus derived only from cultures inoculated with seed virus. The virus in the final vaccine shall not be more than five cell culture passages removed from the virus used in the preparation of a vaccine shown to be immunogenic and safe in man.

3.1.4 Tests on seed lots

The seed lot used for the production of vaccine shall be free from detectable extraneous agents and shall satisfy the requirements specified for single harvests and bulk suspensions given in Part A, sections 3.3, 3.4, 3.5 and 3.6.

3.1.4.1 Tests for neurovirulence. Each seed lot shall be shown to be free from neurovirulence by tests in rubella-susceptible monkeys.

Immediately prior to the test, each monkey should be shown to be serologically negative for rubella. At least 10 monkeys should be employed in each test. The material under test should be given to each monkey by inoculation of 0.5 ml into the thalamic region of each hemisphere. The total amount of rubella virus inoculated into each monkey should be not less than the amount contained in the recommended single human dose of vaccine. Monkeys should be observed for 17 to 21 days for symptoms of paralysis and other evidence of neurological involvement. Animals that die within 48 hours of injection may be replaced. The test is invalid and should be repeated if more than 20% of the monkeys die from nonspecific causes. At the end of the observation period each monkey should be autopsied and histopathological examinations should be made of appropriate areas of the brain for evidence of central nervous system involvement.

There shall be no clinical or histopathological evidence of involvement of the central nervous system attributable to the inoculated virus.

In some countries the seed lot itself is not tested but may be accepted if each of the first five undiluted clarified virus pools prepared from the same seed lot satisfy the requirements of the test for neurovirulence.

3.2 Control cell cultures

At least 500 ml of the cell suspension at the concentration employed for seeding vaccine production cultures shall be used to prepare control cultures.

The cells set aside as control material shall be treated in a similar manner to the production cell cultures but 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 latest, 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 may 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 required in this section shows evidence of the presence in a control culture of any adventitious agent, the rubella 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 guineapig red cells. If the guineapig red cells have been stored they shall have been stored for not more than seven days at a temperature of 2-8°C.

This test is usually made using guineapig red cells. In some countries the national control authority requires that tests for haemadsorbing viruses be made in addition using other types of red cells including human (blood group IV, O), monkey, and chicken (or other avian red cells). The cultures should be examined at three to five days and again at 12 days. All tests should be read after incubation for 30 minutes at 0-4°C and again after a further incubation for 30 minutes at 20-25°C. In addition the test with monkey cells should be read after a still further incubation for 30 minutes at 34-37°C.

3.2.2 Tests for other extraneous agents

At the time of each harvest of the production cultures and 14 days after the day of inoculation of the production cultures with seed lot virus, a sample of the pooled fluids shall be taken at each period of collection from each group of control cultures. Ten millilitres 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 both human and simian cells.

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 seven 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.

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3.2.3 Additional test on control cells if rabbit kidney cells are used for production

At the time of the last virus harvest from the production cultures a sample of the control cells shall be stained by appropriate methods for the detection of *Nosema caniculi*.

In some countries the Giemsa stain is used, but in others the immunofluorescent technique is preferred because it appears to be more sensitive for the detection of low levels of contamination.

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

3.2.4 Additional tests on control cells if duck 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 the avian leucosis viruses, and liver or kidney cell cultures of embryos are used for detecting the adenoviruses.

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

3.2.5 Additional tests on control cells if human diploid cells are used for production

Cells sufficient for chromosome monitoring (Part A, section 3.2.5.1) and for preparing control cultures (Part A, 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. These cells, or cells subcultured from these cells, shall be used for making preparations for chromosome monitoring. The remaining cells shall be set aside as control material. The serum used for the propagation of the cells shall satisfy the conditions of Part A, section 3.1.2.1.

3.2.5.1 Chromosome monitoring pool—preparation and testing

Preparations shall be made on a representative sample from the pooled cell substrate removed from the culture vessels. Chromosome monitoring shall be done at the stage equivalent to the doubling level at which cells are to be inoculated with vaccine virus, or within three population doublings beyond this stage, but if the cells are subcultivated they shall be repooled. For determination of the general character of the cell material, a minimum of 300 cells shall be examined for frequency
of polyplody and a minimum of 100 metaphase plates for exact counts of chromosomes, and analysis of karyotype shall be performed on at least one selected cell. The metaphase plates shall be examined for characteristics that shall include frequency of chromosome breaks, structural chromosome abnormalities, other abnormalities such as de-spinalization or marked attenuations of the primary or secondary constrictions, and the presence of minute chromosomes.

For vaccine production, examination of the cells is usually made between the 27th and 33rd population doubling. The frequency of cells in metaphase with chromosome breaks should not exceed 5%, with structural abnormalities not more than 4% and with polyplody not more than 5%.

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

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.

Permanent stained slide preparations of the chromosome monitoring pool, or photographs of these, shall be maintained as part of the record of the batch of vaccine and for monitoring successive batches.

It is desirable that a portion of the sample of pooled cell substrate removed from the culture vessels 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 cells that have normal karyology may be used for vaccine production.

3.2.5.2 Test for heterotransplantability. At the end of the observation a test for heterotransplantability shall be done on the control cells as outlined in Part A, section 3.1.2.

Only those cells shown not to be heterotransplantable may be used.

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1 These upper limits correspond to the 99 percentile of the values established from a large number of observations on the cell seed system derived from the human diploid cell strain WI-38. However, greater experience of, and more observations on, a particular cell seed in a number of laboratories may result in revised figures being established for such a percentile. National control authorities should consider all available information in specifying the criteria to be met. For detailed information see: International Association of Microbiological Societies, Cell Culture Committee. Minutes of the eighth meeting; Chatham Bars, MA, USA, 1971, Geneva, International Association for Biological Standardization, 1971, p. 71. The values given on p. 71 will not necessarily be applicable if another human diploid cell strain is used.
3.3 Production of single harvests

3.3.1 Cells used for vaccine production

On the day of inoculation with seed lot virus, each cell culture shall be examined for degeneration caused by an infective agent. If this examination shows evidence of the presence in a cell culture of any adventitious agent, the whole group of cultures concerned shall not be used for vaccine production.

The inoculated cell cultures for vaccine production shall be incubated under controlled temperature conditions approved by the national control authority.

If animal serum is used for the growth of cell cultures before the harvesting of virus then the medium shall be removed and replaced with serum-free maintenance medium, the cells being rinsed before being placed in the new medium. The rinsing shall be such that the serum concentration in the final vaccine shall be not more than one part per million.

In some countries control tests are carried out to detect the residual animal serum in the final vaccine.

Penicillin shall not be used in the cell culture medium.

3.3.2 Harvesting

Harvesting of virus fluid shall be carried out by a method approved by the national control authority. Multiple harvests may be made, in which case the single harvests are stored at 4°C until pooling. No antibiotics shall be added at the time of harvesting.

Each single harvest or pool of multiple harvests shall be tested for sterility and virus content according to the following subsections. Each single harvest or pool of multiple harvests shall be tested according to the provisions of Part A, section 3.3, unless those tests are made on the virus pool.

The samples of single harvests shall be taken for testing at the time of harvesting and if not tested immediately shall be kept at a temperature below −60°C until tested.

3.3.2.1 Sterility tests. A volume of at least 10 ml of each single harvest shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for
Sterility of Biological Substances)\(^1\) as well as for mycoplasma by a method approved by the national control authority.

Tests for mycoplasma should be done using both solid and liquid media that have been shown to be capable of supporting the growth of sterol-requiring and non-sterol-requiring mycoplasmas, and using at least 10 ml of the single harvest for each group of tests.

3.4 Control of virus pools prior to clarification

The virus pool shall be prepared from one single harvest or from a pool of single harvests and shall be submitted to the following tests unless these tests have been done on each single harvest, with the exception that even in that event tests for bacterial and mycotic sterility, according to Part A, section 3.3.2.1, shall also be done on the virus pool.

In those tests requiring prior neutralization of rubella virus, the antiserum used shall not be from the same species as that used to provide cells for vaccine production. The immunizing antigen used for the preparation of the antiserum shall be produced in cell cultures free from extraneous microbial agents that might elicit antibodies inhibitory to the growth of any extraneous agents that may be present in the rubella virus pool.

3.4.1 Test for Mycobacterium tuberculosis

Each virus pool shall be tested for the presence of mycobacteria (human, bovine, and avian) by culture methods appropriate for detection of the organisms most likely to be found in the cell culture system used.

3.4.2 Tests in cell cultures

A volume of each virus pool equivalent to at least 500 human doses (see Part B, section 1) or 50 ml, whichever represents the greater volume, shall be tested for adventitious agents by inoculation into simian cell cultures. Similar volumes of the virus pool shall likewise be tested in human cell cultures and in cell cultures of the type used in the preparation of the virus pool. The cell cultures shall be observed for at least 14 days. Ten per cent of the sample shall be neutralized and tested in human and simian cell cultures as well as in cell cultures of the same species (but not the same cells) as those used for the production of virus.

The virus pool passes the tests if none of the cell cultures shows evidence of the presence of any adventitious agents attributable to the pool.

3.4.3 Test in adult mice

Each virus 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 intraperitoneally with at least 0.5 ml of the virus 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 five additional mice, which shall be observed for 21 days.

The virus 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 virus pool.

3.4.4 Test in suckling mice

Each virus 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 virus 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 virus 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 virus pool.
3.4.5 Test in guineapigs

Each virus pool shall be tested for *Mycobacterium tuberculosis* and other adventitious agents by the intraperitoneal inoculation of 5.0 ml of the virus pool into each of at least five guineapigs of 350–450 g weight. The animals shall be observed for at least 42 days for signs of disease. All guineapigs that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined macroscopically, and the tissues shall be examined both microscopically and culturally for evidence of infection. Animals that survive the observation period shall also be autopsied and examined macroscopically.

The virus pool passes the test if at least 80% of the guineapigs remain healthy and survive the observation period and if none of the animals shows evidence of infection with *Mycobacterium tuberculosis* or any adventitious transmissible agents attributable to the virus pool.

3.4.6 Additional test if rabbit kidney cell cultures are used

If rabbit kidney cell cultures are used for vaccine production the following additional test shall be made.

A minimum of 15 ml of each virus pool shall be tested by inoculation into at least five healthy rabbits, each weighing 1500–2500 g. Each rabbit shall receive intradermal injections in multiple sites of a total of 1.0 ml of the virus pool and a subcutaneous injection of 2.0 ml of the virus pool, and the animals shall be observed for at least 30 days. Each rabbit that dies after the first 24 hours of the test or is sacrificed because of illness shall be autopsied and the brain and organs removed and examined.

The virus pool is satisfactory if at least 80% of the rabbits remain healthy and survive the entire period and if all the rabbits used in the test fail to show lesions of any kind at the sites of inoculation and fail to show evidence of any viral infection.

3.4.7 Additional tests if duck embryo cell cultures are used

If duck embryo cell cultures are used for vaccine production the following additional tests shall be made in eggs or cell cultures derived from flocks free from specific pathogens.

A volume of each virus pool equivalent to at least 50 human doses of vaccine (see Part B, section 1) or 5 ml, whichever represents the greater volume, or proportionate amounts drawn from individual harvests totalling such a volume, shall be tested for avian leucosis viruses by a method approved by the national control authority.
A volume of each virus pool, equivalent to at least 100 human doses of vaccine (see Part B, section 1) or 10 ml, whichever represents the greater volume, shall be tested in a group of embryonated duck 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 0.5 ml of inoculum per egg; 5 ml of the sample inoculated by each route shall be neutralized by specific antiserum.

The virus pool passes the test if there is no evidence of the presence of avian leukosis viruses.

It is desirable that in addition further similar volumes of each virus pool should be tested for avian adenovirus by inoculation of chicken liver cell cultures.

A similar volume of each virus pool shall be inoculated into embryonated duck eggs by the test described in Part A, section 3.4.6.

The virus pool passes the test if there is no evidence of the presence of any adventitious agents attributable to the virus pool when tested according to the requirements of Part A, section 3.4.

3.5 Control of bulk suspension after clarification

3.5.1 Clarification of bulk suspension

The bulk suspension shall be clarified by a method that will remove intact cells.

3.5.2 Sampling

Samples of the clarified bulk suspension shall be taken immediately after clarification. If not tested immediately, the samples shall be kept at a temperature below 

3.5.3 Identity test and virus content

The rubella virus in the clarified bulk suspension shall be serologically identified and the virus content determined by cell culture titrations using a reference preparation of live rubella virus for comparison (see Part A, section 1.3).

3.6 The final bulk

The operations necessary for preparing the final bulk shall be conducted in such a manner as to avoid contamination of the product.

The dilution and mixing procedures involved in preparing the final bulk should be those approved by the national control authority.
3.6.1 *Added substances*

Any substance that is added to the product in preparing the final bulk shall have been shown to the satisfaction of the national control authority not to impair the safety and efficacy of the vaccine in the concentration used.

4. **FILLING AND CONTAINERS**

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

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

5. **CONTROL TESTS ON FINAL PRODUCT**

Samples shall be taken from each filling lot for the tests in the following sections.

5.1 **Identity test**

The virus in the final containers shall be identified by appropriate methods.

5.2 **Tests for bacteria and fungi**

Liquid vaccine shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5, of the Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances).²

5.3 **Virus concentration**

The virus content in each of at least three ampoules selected at random from each drying lot shall be determined individually. This determination shall be made in terms of PFU per ml and/or in terms

of TCID$_{50}$ per ml in parallel with the determination of the virus concentration of a reference preparation of rubella virus of known virus titre (see Part A, section 1.3). The determination of the number of PFU per ml shall be based on a total count of at least 100 clearly defined plaques on at least three different cell sheets per dilution. The determination of the number of TCID$_{50}$ per ml shall be based on the use of tenfold dilution steps with 10 tubes per dilution, or any other arrangement of dilutions and tubes yielding equal precision.

The detailed procedures for carrying out this test and for interpreting the results should be those approved by the national control authority. In some countries tests are made in microtitre cultures rather than tubes. In this case the virus titre per millilitre may be calculated and should be used.

The virus content of each ampoule shall be not less than 10$^7$ TCID$_{50}$ or 10$^5$ PFU per human dose.

5.4 Innocuity tests

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

5.5 Residual moisture

The residual moisture in a representative sample of each freeze-dried lot shall be determined by a method approved by the national control authority. The moisture content shall be not greater than 2%.

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) $^1$ 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) $^2$ 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) ¹ shall apply, with the addition of the following.

The label on the container shall include the following information:
— the name of the strain of rubella virus contained in the vaccine.

The leaflet accompanying the package shall include the following information:
— the name of the strain of rubella virus contained in the vaccine;
— the name of the cell cultures in which the vaccine was prepared;
— the nature and amount of any preservative or stabilizer present in the vaccine;
— the amount of virus contained in one recommended human dose;
— a statement that after the vaccine is reconstituted it should be used without delay, or if not used immediately stored between 2°C and 8°C for a period not exceeding eight hours;
— a statement that the vaccine must not be given to a pregnant woman and that a woman must not become pregnant within two months of having the vaccine.

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) ¹ 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 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

continuously at a temperature below –20°C. 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 virus content of the human dose for a period equal to that between the date of issue and the expiry date. The manufacturers shall have shown by accelerated degradation tests that the vaccine in the freeze-dried form is stable.

10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory test for virus concentration, the date of this test being that on which the test system was inoculated. The expiry date shall not be more than two years after the date of the last satisfactory test for virus concentration.

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) \(^1\) shall apply.

The national control authority shall give directions to manufacturers concerning the rubella virus strains to be used in vaccine production and concerning the recommended human dose.

The national control authority should take into consideration all information available on strains before deciding on those permitted for vaccine production.

In addition the national control authority shall provide a reference preparation of live rubella virus (see Part A, section 1.3) for tests for virus concentration (see Part A, sections 3.5.3 and 5.3), and shall specify the requirement for virus content that shall be fulfilled in order to achieve adequate immunization of humans using the recommended human dose.

---

2. RELEASE AND CERTIFICATION

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

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

The purpose of the certificate is to facilitate the exchange of live rubella vaccine between countries.
Appendix

SUMMARY PROTOCOL OF RUBEOLA VACCINE PRODUCTION
Based on the Requirements for Rubella Vaccine (Live)
(Requirements for Biological Substances No. 24)

Name of manufacturing laboratory
Address
Lot number of vaccine
Number of freeze-drying lot
Date of initiation of last potency test
Expiry date
Proprietary name of vaccine
Number and volume of ampoules or vials in the lot

Seed virus

Seed virus strain
Date when established
Seed lot reference number
Date(s) of satisfactory neurovirulence test(s)

Cell source

Cell used for production
Tests for freedom from pathogenic microorganisms of cloned colony or cell seed ampoules, at the time of preparation of cell cultures

Certified satisfactory
Date
Signature of head of laboratory

80
Tests on control cells for freedom from microorganisms

<table>
<thead>
<tr>
<th></th>
<th>Quantity or volume put on test</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Quantity or volume passing test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of haemadsorbing viruses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tests in simian cell cultures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tests in human cell cultures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tests in type of cells used in production</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production in rabbit kidney cells: Test for absence of <em>Nosema caniculi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production in duck embryo cells: Test for absence of avian leucosis viruses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Production in human diploid cells: Tests for karyology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cells examined</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of abnormalities found</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for heterotransplantability</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method used</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals on test</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date of start of test</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date of end of test</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did any animal show a nodule? If so how many and for how long?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of control animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number showing tumours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Signature of head of laboratory: ________________________________

81
Test on single virus harvests

<table>
<thead>
<tr>
<th>Sterility</th>
<th>Volume tested</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test for bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for fungi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for mycoplasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus titre</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Test on bulk harvests before clarification

<table>
<thead>
<tr>
<th>Test for M. tuberculosis</th>
<th>Volume tested</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Volume passing test</th>
</tr>
</thead>
</table>

Simian cell cultures:

(a) Neutralized virus
(b) Unneutralized virus

Human cell cultures:

(a) Neutralized virus
(b) Unneutralized virus

Cell type used for production:

(a) Neutralized virus
(b) Unneutralized virus

Tests for extraneous agents in animals:

<table>
<thead>
<tr>
<th>Animal</th>
<th>Volume tested</th>
<th>No. of animals inoculated</th>
<th>No. survived</th>
<th>Period of survival</th>
<th>Volume passing test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suckling mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea pigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

82
**Tests in embryonated chicken eggs:**

<table>
<thead>
<tr>
<th>Volume tested</th>
<th>No. of egg inoculated</th>
<th>No. survived</th>
<th>Period of survival</th>
<th>Volume passing test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Neutralized virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) Unneutralized virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Test for vaccine prepared in rabbit kidney cells**

<table>
<thead>
<tr>
<th>Volume tested</th>
<th>No. of rabbits inoculated</th>
<th>No. survived</th>
<th>Volume passing test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Test for vaccine prepared in duck embryo cell culture (avian leukosis viruses)**

<table>
<thead>
<tr>
<th>Volume tested</th>
<th>No. of eggs inoculated</th>
<th>No. survived</th>
<th>Volume passing test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Test in embryonated duck eggs:**

<table>
<thead>
<tr>
<th>Volume tested</th>
<th>No. of eggs inoculated</th>
<th>No. survived</th>
<th>Volume passing test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Neutralized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) Unneutralized</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Signature of head of laboratory  

**Tests on bulk suspension after clarification**

<table>
<thead>
<tr>
<th>Result</th>
<th>Date completed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for removal of cells</td>
<td></td>
</tr>
<tr>
<td>Identity test</td>
<td></td>
</tr>
</tbody>
</table>
| Sterility tests:
  Tests for bacteria |                     |
  Tests for fungi |                     |

Signature of head of laboratory  

83
Tests on final vaccines

<table>
<thead>
<tr>
<th>Identity test</th>
<th>Result</th>
<th>Date completed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterility tests:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tests for bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tests for fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus concentration per human dose (log_{10} ID_{50} or PFU)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inocuity:</th>
<th>No. of animals inoculated</th>
<th>No. survived</th>
<th>Period of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Guinea pigs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Residual moisture
Specify method used
% moisture

Signature of head of laboratory

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

Signature
Annex 4

REQUIREMENTS FOR BRUCELLA MELITENSI S STRAIN
REV. 1 VACCINE (LIVE — FOR VETERINARY USE)
(Requirements for Biological Substances No. 25)

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INTRODUCTION

Brucellosis in goats and sheep caused by *Brucella melitensis* is widespread in many areas of the world. It is responsible for economic losses through interference with breeding programmes and reduction in milk yield. The disease is transmissible to man and because of the greater pathogenicity of *B. melitensis* than of *B. abortus* for man the organism constitutes a greater hazard to public health. Transmission of this infection from sheep and goats to cattle occurs, producing an infection that follows a course similar to that of *B. abortus* infection but characterized often by a lack of clinical symptoms. Thus, such infected cattle are an even greater danger to man because of the greater pathogenicity of *B. melitensis*. 

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Since 1955 investigations in Iran, Israel, Italy, Malta, Mexico, South Africa, Spain, and the USA, have shown that a strain of *B. melitensis* of reduced virulence, designated strain Rev. 1, is highly effective in the control of *B. melitensis* infection in goats, sheep, and cattle when used as a live vaccine. Strain Rev. 1 was isolated as a non-streptomycin-dependent clone from a population of streptomycin-dependent cells that were in turn derived from the parental virulent strain 6056 of *B. melitensis*. The WHO Expert Committee on Biological Standardization at its meeting in Geneva in November 1970 agreed that there was a need for the formulation of requirements for the control of *B. melitensis* Rev. 1 vaccine.

The following international requirements for *B. melitensis* strain Rev. 1 vaccine (live for veterinary use) have been fitted into the framework adopted in the Requirements for Biological Substances Nos. 1–23 already published by WHO,\(^1\) and in drafting them account has been taken of the opinions of consultants, the regulations and requirements for the manufacture and control of *Brucella* vaccines that have been formulated in a number of countries, and information from both published and unpublished reports. In addition, opinions and data have been received from a number of experts and institutions, whose assistance is gratefully acknowledged below:

Dr G. G. Alton, Division of Animal Health, Animal Health Research Laboratory, Commonwealth Scientific and Industrial Research Organization, Parkville, Victoria, Australia

Dr A. Ceccharelli, Institute of Infectious Diseases, Prophylaxis, and Veterinary Hygiene, Pisa, Italy

Mr J. Davidson, Head, Biological Products and Standards Department, Central Veterinary Laboratory, Weybridge, Surrey, England

Professor S. S. Elberg, School of Public Health, University of California, Berkeley, CA, USA

Professor R. Farina, Director, Institute of Infectious Diseases, Prophylaxis, and Veterinary Hygiene, Pisa, Italy

Dr L. M. Jones, Department of Veterinary Science, University of Wisconsin, Madison, WI, USA

Dr M. Kaveh, Director-General, Razi State Serum and Vaccine Institute, Tehran, Iran

Dr W. J. B. Morgan, Deputy Director, Central Veterinary Laboratory, Weybridge, Surrey, England

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GENERAL CONSIDERATIONS

The characteristics of *B. melitensis* strain Rev. 1 are (1) its stable and reduced pathogenicity for animals together with a relatively high immunogenicity for goats and sheep; (2) its inability to spread from animal to animal; (3) its slow growth on solid media, requiring four days at 37°C to produce colonies 1–2 mm in size; (4) the small size of its colonies (1–2 mm); (5) inhibition of growth by 1 : 50,000 thionine and 1 : 50,000 basic fuchsins; (6) its failure to grow on agar containing 5–10 units of penicillin per ml; and (7) its ability to grow on agar containing 2–5 μg/ml of streptomycin.

The degree of attenuation of strain Rev. 1 for animals is demonstrated in guineapigs of the Hartley strain. When inoculated with graduated doses (10⁶–10⁹) the animals clear their tissues of organisms detected by culture methods in 3–5 months whereas virulent strains of *B. melitensis* persist in such animals for 6–12 months. Various breeds of goats clear their tissues (usually within 3–4 months) of receiving 10⁹ Rev. 1 cells inoculated subcutaneously, and fat-tailed sheep clear their tissues rapidly. Occasionally a goat or sheep will excrete the organism in its milk for a variable length of time but the numbers of strain Rev. 1 present on such occasions have been shown not to be able to establish infection in monkeys given one feeding of 10⁶ cells of Rev. 1 or nine weekly feedings of 10⁶ cells per week by stomach tube. Although vaccination of sheep and goats can cause abortion, several serial passages through pregnant animals did not cause changes in the degree of attenuation for guineapigs or pregnant goats and sheep, and animals in contact with aborting sheep did not become infected.¹

These characteristics are maintained by the careful selection of seed cultures, the preparation of an original seed with the desired properties for vaccine production, and its preservation in the freeze-dried form. Preparation of the vaccine itself in freeze-dried form is to be preferred since freeze-dried preparations are more stable. Consideration should be given to providing an international reference preparation of *B. melitensis* strain Rev. 1 vaccine.

Goats and sheep are usually inoculated at the age of 4–6 months with a dose of vaccine containing the recommended number of viable organisms. One injection is known to protect goats for 4–5 years. The animal is, in effect, protected for the rest of its life and produces an

agglutinin response that, in 70% of animals, becomes negative within one year and a transient complement-fixation reaction that often disappears within six months. Fifteen-month-old sheep given Rev. 1 vaccine before their first breeding were resistant to natural exposure in their third pregnancy; lambs vaccinated at four months of age were resistant when similarly challenged in their second pregnancy but the immunity in such cases may become low by the third pregnancy in sheep. The routine vaccination of all goats and sheep with the full dose of Rev. 1 (10⁶ cells) before they reach breeding age is the most effective way to achieve control by vaccination, although in the initial stages of a campaign it may be of use to vaccinate adult goats with a reduced dose (e.g., 10⁵ cells). Pregnant animals should not be vaccinated and it is advisable not to vaccinate lactating animals except in the late stage of lactation.

Problems of dissociation are liable to develop in Rev. 1 production but may be avoided by careful colony selection in the production of vaccine seed material ¹ and by filtering instead of autoclaving the non-agar portions of the medium, where this is practical.

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.

**PART A:**

**MANUFACTURING REQUIREMENTS**

1. DEFINITIONS

1.1 International name and proper name

The international name shall be *Brucella melitensis* strain Rev. 1 vaccine (live — for veterinary use) or the equivalent 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

_Brucella melitensis_ strain Rev. 1 vaccine (live— for veterinary use) is a liquid or freeze-dried preparation of live _B. melitensis_ strain Rev. 1. The preparation shall satisfy all the requirements formulated below.

_Brucella melitensis_ strain Rev. 1 vaccine (live—for veterinary use) is referred to in this document as “Rev. 1 vaccine.”

1.3 International standards, international reference preparations, and international units

Since no international standard or reference preparation of Rev. 1 vaccine is yet available, no requirements based on comparisons with such preparations can at present be formulated.

1.4 Terminology

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

**Original seed.** A quantity of live _B. melitensis_ strain Rev. 1 processed together, adequately characterized, of uniform composition, and maintained in the freeze-dried form, which has been shown to be safe and effective on parenteral administration to guineapigs of the Hartley strain.\(^1\) It may be used for vaccine production or for the preparation of seed lots for vaccine production.

**Seed lot.** A quantity of live _B. melitensis_ strain Rev. 1 processed together and of uniform composition and maintained in the dried form or in liquid nitrogen. A seed lot is prepared from an original seed of live _B. melitensis_ strain Rev. 1 and is not more than three passages removed from the original seed. A seed lot possesses the colonial and other characteristics typical of the original seed as determined by conventional and definitive typing procedures, by reactivity tests in animals, and by antigenicity and immunogenicity tests (see Part A, section 5).

**Single harvest.** A suspension of bacteria harvested on the same day from a number of cultures that have been seeded with material from an original seed or from a single seed lot and incubated together and are not more than three passages removed from the original seed or from the seed lot, whichever is applicable.

---

\(^1\) A satisfactory original seed of _B. melitensis_ strain Rev. 1 in a form for preparing vaccine or for preparing seed lots may be requested through the World Health Organization or obtained directly from Professor S. S. Elberg, School of Public Health, University of California, Berkeley, CA 94720, USA (see Part A, section 3.1.2).
Final bulk. The finished material derived from a single harvest or a pool of a number of single harvests and present in the container from which the final containers are filled.

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

2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in Part A of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)¹ shall, in principle, apply to establishments manufacturing Rev. 1 vaccine.

3. PRODUCTION CONTROL

3.1 Control of source materials

3.1.1 B. melitensis strain

The B. melitensis strain Rev. 1 used for preparing an original seed shall be one that has been shown to yield a vaccine that is safe and effective in protecting goats and sheep against brucellosis.

3.1.2 Seed lot system

The production of Rev. 1 vaccine shall be based on the seed lot system using material derived from an original seed or a seed lot prepared from such material (see Part A, section 1.4).

Original seed is usually subcultured on tryptcase soy agar slants or another suitable medium and incubated at 37°C for 48-96 hours before being distributed. The slants may be stored at 5°C ± 3°C for a period not exceeding two weeks before being subcultured for vaccine preparation or for the preparation of a seed lot.

Seed lots prepared from an original seed and fulfilling the requirements in both this and the following section shall be used only for vaccine production; they shall not be used for the preparation of further seed lots.


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3.1.3 Tests on seed lots and seed

A seed lot shall be characterized by conventional and definitive typing procedures and shall be tested for reactivity in guineapigs and for antigenicity and immunogenicity according to the recommendations given in Part A, sections 5.5 and 5.6. It shall fulfill the criteria for those characterizations and shall meet the requirements for any tests specified by the national or regional control authority.

It is desirable that characterization of a seed lot by conventional and definitive typing procedures should be performed in parallel with an original seed.

Seed used for vaccine production, whether prepared from an original seed or from a seed lot, shall be tested for bacterial contamination and for dissociation according to the requirements given in Part A, sections 3.4.2 and 5.3.

3.2 Production precautions

The general production precautions, as formulated in Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories), shall apply in principle to the manufacture of strain Rev. 1 vaccine.

3.3 Control of single harvest

Single harvests shall be prepared by growing B. melitensis strain Rev. 1 on a suitable solid medium incubated in an inverted position or in a liquid culture medium. The liquid culture medium shall be adequately aerated.

A single harvest or a pool of a number of single harvests may be used in preparing the final bulk.

If the final bulk is made from a pool of a number of single harvests, only single harvests derived from the same seed lot shall be used for the production of such final bulk.

If a stabilizer is added to the material, such a stabilizer should have been shown to the satisfaction of the national or regional control authority not to impair the safety and efficacy of the vaccine.

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1 The characteristics of strain Rev.1 are described in Alton, G. G. et al. Laboratory techniques in brucellosis, 2nd ed. Geneva, World Health Organization, 1975 (Monograph Series, No. 55). They are also summarized in the section entitled "General considerations" in these Requirements, pp. 87-88.
3.4 Control of final bulk

3.4.1 Test for bacterial content

An estimation of the bacterial content of the material may be made by a suitable method (e.g., by determination of opacity) in order to adjust the volume for preparing the final bulk so that the number of viable organisms in the final product will meet the requirements given in Part A, section 5.4.

3.4.2 Test for absence of contaminating microorganisms

The final bulk shall be tested for bacterial and mycotic contamination in accordance with the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances)\(^1\) and shall not be used for vaccine production unless it is shown to be free from such contamination.

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)\(^2\) shall apply in principle.

Multiple-dose containers may be used.

5. CONTROL TESTS ON FINAL PRODUCT

If the final product is in the freeze-dried form, all tests made shall be on the vaccine reconstituted to the form in which it is to be used.

5.1 Identity test

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

The identity of each filling lot of vaccine should be verified by the morphological appearance of the bacilli in stained smears or by the characteristic appearance of the growth on media. Suitable culture media are trypticase soy agar and trypticase soy broth.

---

5.2 Test for absence of contaminating organisms

Samples from each filling lot shall be tested for bacterial and mycotic contamination according to the requirements given in Part A, section 5, of Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).\(^1\)

5.3 Test for dissociation

A sample of each filling lot shall be examined for dissociation by culturing on a suitable medium so as to obtain areas containing confluent growth as well as discrete colonies. The filling lot passes the test if no fewer than 95% of the colonies are of the smooth variety.

The vaccine is appropriately diluted and inoculated on to glycerol-dextrose or tryptase soy agar medium in Petri dishes.

After incubating the dishes at 37°C in the inverted position for 96–120 hours, the colonies are checked for colonial morphology by examination with a stereomicroscope and by staining with crystal violet.\(^2\)

5.4 Test for number of viable organisms

The number of viable organism in each filling lot shall be determined by plating suitable dilutions on an appropriate medium.

Suitable media for this purpose are: tryptase soy agar, glycerol-dextrose agar, altini agar, or brucella agar.

It is desirable that the method used should be one that permits an estimate of the error by a standard statistical method.

The recommended dose for goats and sheep shall contain between \(10^8\) and \(2 \times 10^8\) viable organisms, but adult goats should receive a reduced dose, e.g., \(10^6\) cells (see p. 88).

The criteria of precision should be those specified by the national or regional control authority.

5.5 Test for reactivity in guineapigs

It is advisable to test for reactivity in the Hartley strain of guineapig. The test is based on the limited multiplication of 3000 cells of strain Rev. 1 introduced subcutaneously in the groin, and the subsequent clearance of the spleen within 12 weeks of inoculation.

---


It is advisable that this test be carried out parallel with a suitable reference preparation approved by the national control authority.

5.6 Tests for antigenicity and immunogenicity

It is desirable that tests for antigenicity and immunogenicity should be performed on samples of the final product.

The test for antigenicity may be performed by injecting vaccine subcutaneously into guineapigs, using a dose of 3000 cells, to determine the agglutinogenic properties of the vaccine by means of the standard agglutination test or other suitable tests such as the passive haemagglutination test.

When tested 14 days after vaccination, guineapigs should show an antibody response as measured by the standard agglutination test of not less than 320 and not more than 1280 International Units per ml of serum.

The test for immunogenicity may be performed in guineapigs by inoculating 10 animals subcutaneously with 3000 cells of the vaccine and 12 weeks later challenging them subcutaneously on the opposite side with 2000 cells of a virulent strain of B. melitensis such as strain 6015 or strain H38. Six weeks after challenge 90% of the immunized animals should be completely free of the challenge infection. At least six unvaccinated control guineapigs should be challenged with the same challenge dose and all of them should show infection.

It is advisable that the tests should be made in parallel with a suitable reference preparation approved by the national control authority.

5.7 Stability test

Selected filling lots shall be tested for stability by a method approved by the national or regional control authority.

The test should be designed to obtain experimental evidence on which to base the statements concerning storage temperature and expiry dates that appear on the label and the leaflet as required in Part A, section 8. It should involve the determination of the number of viable organisms before and after the samples have been held at appropriate temperatures and for appropriate periods.

As a guide to stability, the residual moisture content of each filling lot of freeze-dried vaccine may be determined. The method of determination should be one approved by the national or regional control authority.
6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)\(^1\) 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)\(^2\) 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)\(^3\) shall apply, with the addition of the following.

If the vaccine is in the freeze-dried form, the leaflet accompanying the package shall include statements (1) that the vaccine shall be kept cool and used within six hours of reconstitution and (2) that injection into pregnant sheep and goats may result in abortion.

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)\(^4\) 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)\(^5\) shall apply.

10.1 Storage conditions

Rev. 1 vaccine shall be protected from light and stored at a temperature of 5°C ± 3°C.

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10.2 Expiry date

The expiry date for refrigerated liquid vaccine shall be not more than four weeks from the earliest date of any single harvest used in production of the vaccine and not more than two weeks from the date of issue of the vaccine. The expiry date for freeze-dried vaccine shall be that approved by the national or regional control authority and shall be based on the results of the stability tests referred to in Part A, section 5.7. The expiry date shall be not more than 18 months from the date of lyophilization of the filled final material.

PART B:
NATIONAL CONTROL REQUIREMENTS

1. GENERAL

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

The national control authority shall give directions to manufacturers concerning the provision and use of the B. melitensis strain Rev. 1 seed to be used in vaccine production and only seed conforming to the requirements of Part A, section 3.1., shall be approved for such use.

The national control authority should satisfy itself from the results of tests on a series of consecutive vaccine lots that the manufacturer is able to reach a satisfactory consistency of quality of the product.

It is desirable that the national control authority should give directions to manufacturers concerning the provision of a reference preparation of B. melitensis strain Rev. 1 for use in the tests for reactivity in guineapigs, for antigenticity, and for immunogenicity (see Part A, sections 5.5 and 5.6).

2. RELEASE AND CERTIFICATION

A vaccine lot 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 lot of vaccine in question meets all national or regional requirements as well as Part A of the present requirements. The certificate shall, in addition, state the date of the last satisfactory test for number of viable organisms, if applicable, the date of lyophilization of the product, 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 release document shall be attached.

The purpose of the certificate is to facilitate the exchange of B. melitensis strain Rev. 1 vaccines between countries.
Annex 5

REQUIREMENTS FOR ANTIBIOTIC SUSCEPTIBILITY TESTS

I. AGAR DIFFUSION TESTS USING ANTIBIOTIC SUSCEPTIBILITY DISCS ¹

(Requirements for Biological Substances No. 26)

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Appendix 1. Calculation of disc content from assay data 125

Appendix 2. Criteria for interpretation based on the Bauer-Kirby procedure 127

¹ Prepared by the following members of the WHO Secretariat: Dr. D. Barua, Medical Officer, Bacterial Diseases, WHO, Geneva, Switzerland; Dr. V. A. Chabbert, Medical Bacteriologist, Pasteur Institute, Paris, France (Consultant); Dr. L. Houang, Medical Officer, Health Laboratory Technology, WHO, Geneva, Switzerland; Dr. J. W. Lightbown, Head, Division of Antibiotics, National Institute for Biological Standards and Control, London, England (Consultant); Dr. F. T. Perkins, Chief, Biologicals, WHO, Geneva, Switzerland; Dr. W. B. Wright, Deputy Associate Director for Pharmaceutical Research and Testing, Bureau of Drugs, Food and Drug Administration, Washington, DC, USA (Consultant).
INTRODUCTION

The need for international standardization of procedures for determining the susceptibility of microorganisms to antibiotics has been recognized since 1961, when the WHO Expert Committee on Antibiotics made recommendations to this end. The results of an extensive series of international collaborative studies of different techniques of testing using broth dilution, agar dilution, and agar diffusion, organized in conjunction with WHO, were published by Ericsson & Sherris in 1971. While agreement could not be reached internationally on many procedural details of experimental technique and interpretation, it was agreed that standardization of the reagents used in the testing was desirable and possible. Agar diffusion procedures are the methods most widely used internationally. Antibiotic susceptibility discs are manufactured in only a few countries, however, and international requirements defining their quality and performance would be helpful to those countries dependent on importing these biological materials or considering manufacturing them in the future. The safety of the patient and the effectiveness of treatment may well depend as much on an assurance of the quality of the antibiotic susceptibility discs and on their method of use as on the quality and quantity of the antibiotic preparation chosen for therapy.

Grateful acknowledgement is made to the experts and institutions listed below for their comments and advice and for supplying additional data relevant to these requirements:

Dr P. Actor, Research and Development Division, Smith Kline and French Laboratories, Philadelphia, PA, USA
Mr B. Arret, Deputy Director, National Center for Antibiotics Analysis, Pharmaceutical Research and Testing, Food and Drug Administration, Washington, DC, USA
Dr A. Balows, Director, Bacteriology Division, Bureau of Laboratories, Center for Disease Control, Atlanta, GA, USA
Dr J. Bang, Head, Department of Antibiotics, State Serum Institute, Copenhagen, Denmark
Dr L. Baquerizo Amador, Miguel H. Alcivar Clinic, Guayaquil, Ecuador
Mrs J. Batty, Secretary, Standardization Committee, International Union of Immunological Societies, West Wickham, Kent, England

Mr D. F. J. Brown, Public Health Laboratory, Department of Microbiology, Central Middlesex Hospital, London, England
Dr L. Chambon, Deputy Director, Pasteur Institute, Paris, France
Dr W. K. Chang, Medical and Health Department, Institute of Pathology, Jockey Club Clinic, Hong Kong
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Dr A. El Kholy, Director, Rheumatic Fever Project, Cairo, Egypt
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Dr L. Hig-Mandic, Department of Biological Standardization, Institute of Immunology, Zagreb, Yugoslavia
Mr P. Isaacson, Antibiotics Laboratory, National Biological Standards Laboratory, Canberra, A.C.T., Australia
Dr I. Jod, “Human” Institute for Serobacteriological Production and Research, Budapest, Hungary
Dr C. Leriche, Director, National Institute of Public Health, Oslo, Norway
Dr A. Mainten, Head, Department of Chemotherapy, National Institute for Public Health, Bilthoven, Netherlands
Dr A. G. Mathews, Consultant (Standardization), Commonwealth Serum Laboratories, Parkville, Victoria, Australia
Professor S. M. Navashin, Corresponding Member of the USSR Academy of Medical Sciences, Director of the National Research Institute of Antibiotics, Moscow, USSR
Dr R. Norton, Microbiologist, Division of Anti-Infective Drug Products, Bureau of Drugs, Food and Drug Administration, Washington, DC, USA
Dr A. J. Oliver, Chairman and Managing Director, Mast Laboratories Limited, Liverpool, England
Dr A. S. Oustchoorn, Senior Scientist, United States Pharmacopeia, Rockville, MD, USA
Professor A. Pauch, Director, Bacteriological Control Section, National Health Laboratory, Ministry of Health, Montpellier, France
GENERAL CONSIDERATIONS

In formulating these requirements it has been possible to be specific about many of the details of the production and control of antibiotic susceptibility discs, and these details are included in Part A of the requirements. Although there is no universal agreement on the quantity of antibiotic that should be contained in each disc it has been possible to indicate the quantities commonly used. Furthermore an international code for the identification of the discs has been suggested.
Perhaps the greatest differences between countries concern the details of performing disc susceptibility tests because different media and test conditions are used without a common point of reference, e.g., reference strains. In these requirements attempts have been made to overcome these differences by providing guidance on the directions necessary for the use of the discs and indicating how individual laboratories could achieve greater uniformity by daily performance testing using reference strains (see Part C). Interpretation of the zone diameters has been a constant problem in many countries. The aim of the test is to assess the susceptibility or resistance of an organism to a particular antibiotic in order that the physician may successfully treat the infection. This implies that the level of antibiotic to which the organism is susceptible can be reached in the body at the site of infection. Although there is no international agreement on such levels, some guidance must be given, especially for those countries without much experience in this technique. Therefore, a table of zone diameters has been included indicating whether the organism is resistant, intermediate, or susceptible. This table has been shown to be useful in one country and is of general value if considered in relation to the recommended use of reference strains.

Finally, because the composition of the medium plays such a significant part in the zone sizes obtained, the requirements for a medium (Mueller-Hinton) known to be successful and widely used by many laboratories have been included. A performance test is also included that would permit the use of other media (see Part D).

These requirements apply only to single discs. Multi-discs were considered by the WHO Expert Committee on Antibiotics ¹ to be inferior, and there are no known national requirements for their control.

PART A:
MANUFACTURING REQUIREMENTS FOR SUSCEPTIBILITY DISCS

1. DEFINITIONS

1.1 International names and proper names

The international name of each kind of disc containing a particular antibiotic shall be the International Nonproprietary Name (INN) of

the antibiotic contained therein followed by the words "Susceptibility Disc". 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 antibiotic susceptibility discs that satisfy the requirements formulated below.

1.2 Descriptive definition

Antibiotic susceptibility discs are round flat absorbent discs of uniform diameter and thickness containing an evenly distributed amount of antibiotic.

Antibiotic susceptibility discs commonly have a diameter chosen within the range 5-7 mm. Paper is the most common material from which the discs are made.

1.3 International standards, international reference preparations, and international units

The potency tests described in Part A, section 5.1, provide for the preparation of the standard discs by the laboratory performing control tests. National control authorities shall provide reference preparations of antibiotics for preparing standard discs or for determining the potency of local reference preparations of antibiotics to be used in preparing standard discs.

No international reference preparations of antibiotic susceptibility discs are available.

International standards and reference preparations of antibiotics are in the custody of the National Institute for Biological Standards and Control, London. Samples are distributed free of charge on request to national control authorities. The international standards and reference preparations are intended for the calibration of national standards.

1.4 Terminology

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

Disc. An antibiotic susceptibility (sensitivity) disc containing a specific antibiotic for the determination of the susceptibility of microorganisms to the particular antibiotic.

Batch. A quantity of discs produced during a given cycle of manufacture. The essence of a batch is its homogeneity.

Sample disc. A disc selected from a given batch for quality control tests.

Standard disc. A disc that has been prepared by a quality control laboratory, that contains a specified amount of an antibiotic, and that is intended for use in the assay of the content of a sample disc taken from a particular batch.

Assay organism. A specific strain of microorganism used for the assay of particular antibiotic susceptibility discs.

Culture medium. Microbiological medium for propagating assay organisms.

Assay medium. Microbiological medium used for preparing base layers and/or seed layers in Petri dishes used for assaying the content of discs.

Zone of inhibition. The clear circular area around a disc at the completion of an assay. The size of the zone is expressed as its diameter, which includes the diameter of the disc.

1.5 Contents of antibiotic in discs

The antibiotic content of discs shall be such that when used as described in Part C, section 3, inhibition zones are obtained with organisms against which the antibiotic may be expected to be clinically effective. The minimum inhibitory concentration (see Part C, section 1) shall be within the range of concentrations likely to be obtained in body tissues and fluids during therapy.

Zone diameters should not exceed 40 mm with commonly tested highly susceptible microorganisms encountered in clinical practice and preferably should not exceed 30 mm.

For most antibiotics the application of these guidelines shows that discs of a single antibiotic content can be employed. Some antibiotics have such low toxicity that infections with organisms having minimum inhibitory concentrations with a range of several hundredfold may be amenable to treatment with different dosage schedules. For such antibiotics more than one disc content may be required.

Within these guidelines, there is no precisely correct nominal disc content. Selection of the content to be used is arbitrary within certain limits and is based in part on the logic and convenience of having similar disc contents within a family of antibiotics.
The following disc contents have been shown to be suitable for some families of antibiotics and for some individual antibiotics:

- the penicillinase-sensitive penicillins
  - (e.g., benzylpenicillin) 6 µg
- the penicillinase-resistant penicillins
  - (e.g., meticillin) 5 µg
  - the cephalosporins * 30 µg
  - the aminoglycosides * 10-30 IU
  - the tetracyclines * 30 IU
  - the macrolides * 15 IU
  - the lincomycins * 2-15 IU
  - the polymyxins * 300 IU
  - ampicillin 10 µg
  - bacitracin 10 IU
  - carbenicillin 100 µg
  - chloramphenicol 30 µg
  - novobiocin 30 IU
  - rifampicin 5 µg
  - vancomycin 30 IU

* For purposes of routine testing of clinical isolates for susceptibility to antibiotics, it may be satisfactory to use one type of antibiotic susceptibility disc as a representative of its class of family. However, testing with individual members of the family may be desirable under certain circumstances.

1.6 Codes

Each disc shall be imprinted with three letters to identify the antibiotic contained therein.

The purpose of the three-letter abbreviations is to identify the discs. The abbreviations should not be used in publications, and in any written communication the names of antibiotics should be given in full.

The identification letters should consist of the first three letters of the International Nonproprietary Name, except when confusion may arise by virtue of the existence of several antibiotics with an identical initial stem or when such letters may be undesirable for other reasons.

For the sake of uniformity, the following abbreviations are suggested:

- amikacin AKN
- ampicillin AMP
- bacitracin BAC
- benzylpenicillin PEN
- carbenicillin CAR
- cefalexin CEX
- cefadroxil CFX
- cefalotin CTF
- cefazolin CZL
cefotaxim
chloramphenicol
cloxacillin
clexobacillin
gentamicin
gentamycin
gentamycin
metacillin
minocycline
neomycin
novebicin
oleandomycin
polymyxin B
rifampicin
rifampicin
tobramycin
tobramycin
vancomycin

No numbers should be placed on the discs to indicate the quantity of antibiotic in them, except in the case of specific antibiotics for which two contents may be necessary (see Part A section 1.5).

2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in the WHO "Good Practices in the Manufacture and Quality Control of Drugs" \(^1\) shall apply.

3. PRODUCTION CONTROL

3.1 Control of source material

3.1.1 Antibiotics

The antibiotics to be used in preparing discs shall be of the quality used in pharmaceutical manufacture and shall meet the requirements, if any, of the International Pharmacopoeia, except those for undue toxicity and residue on ignition.

3.1.2 Paper or other material used for preparing discs

The material used for preparing the discs and the method of manufacture shall be such that the discs are uniform and of sufficient thickness to ensure ease of handling. The material shall also permit complete

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and uniform adsorption of a sufficient volume of antibiotic solution and under the conditions of use shall allow uniform release of the antibiotic. When response lines are prepared for each antibiotic, using discs made from the material and the assay procedures described in Part A, section 5.2, the slopes of the lines shall be comparable to those obtained with the response lines of the respective standard antibiotic discs. The material shall be tested and shown to be free from inherent inhibitory activity and from manufacturing residues, such as sulfites, which may combine with or inactivate antibiotics.

3.1.3 Solvents

Aqueous or organic solvents used to dissolve the antibiotics or which act as a vehicle for impregnating the paper or other material shall be free from components that have inhibitory activity or may inactivate the antibiotics or affect their diffusion properties.

3.1.4 Inks and colours

Dyes and inks for coding antibiotic discs shall not prevent uniform absorption and distribution of antibiotic in the material, nor produce inhibition zones, nor shall they interfere with, or potentiate, the activity of any antibiotic. They shall persist for the duration of the test.

3.2 Production methods

3.2.1 Preparation of antibiotic solutions

The antibiotic to be used for preparing solutions for impregnating paper or other material shall be weighed accurately and the activity or amount contained therein shall be calculated by multiplying the weight by the potency or purity, respectively, of the antibiotic in international units or micrograms per unit of weight. The antibiotic shall be dissolved in an appropriate volume of one or more solvents having regard for any change in volume that may take place during mixing.

Care should be taken to protect such solutions from factors such as excessive light and heat that are known to cause loss of antibiotic activity.

3.2.2 Uniformity of content

The paper or other material used for preparing discs shall be impregnated uniformly with a solution containing a concentration of antibiotic
activity per unit volume sufficient to ensure that after drying and completion of the manufacture each disc shall contain the required quantity of antibiotic (see Part A, section 1.5).

3.2.3 Drying of impregnated paper or other material

The impregnated paper or other material shall be dried by an appropriate process that removes the solvent without disturbing the uniformity of antibiotic distribution in the material or causing an excessive loss of potency.

The process should be checked by determining the moisture content of representative discs from a batch shown to be satisfactory in stability studies.

4. CONTAINERS

The container shall be a tightly closed container as defined by the International Pharmacopoeia.¹

Containers may be in the form of cartridges that are used for repetitive dispensing of discs. Dispensing devices into which a number of such cartridges are mounted may be used, provided the entire device is provided with a closure making it a tightly closed container.

Each container should contain a desiccant that indicates by a change in colour when it is no longer effective.

5. CONTROL TESTS ON DISCS

5.1 Identity

The identification of the antibiotic contained in a disc requires the application of many different analytical procedures, e.g., chromatography, electrophoresis, spectrophotometry, enzymic inactivation. Such tests are described in various pharmacopoeias but would generally require the antibiotic to be extracted from the disc and concentrated. The character of the edge of the zone of inhibition allows differentiation between some groups of antibiotics but is not sufficiently specific to allow positive identification. The difficulties of identification emphasize the need to ensure the application of good manufacturing practices at the time of production (see Part A, section 2).

5.2 Assay of content

5.2.1 Standard discs

Standard discs are prepared using blank discs of paper having the same diameter as the sample discs and the ability to absorb 2.5 to 3.0 times their weight of distilled water. The paper shall exhibit no inhibitory activity nor contain residual material that affects the activity or pH of the antibiotic solution applied to it.

The national control authority should supply paper suitable for the preparation of standard discs.¹

The blank discs are supported so as to allow free circulation of air around them and a volume of 0.02 ml of an accurately prepared solution of the appropriate reference standard is added to each disc. The concentrations of the solutions are adjusted to allow the desired content of antibiotic to be applied to each disc. At least three different concentrations are used to provide standard discs including (a) the quantity of antibiotic described on the label of the sample and (b) two other quantities, one less and one greater than the labelled quantity by the same interval on a logarithmic scale.

Solutions should be unbuffered and prepared in freshly distilled water, methanol, or mixtures of these.

Examples of suitable quantities for preparing standard response lines in terms of the labelled quantity and the intervals are:
- 50%, 100%, and 200% (intervals of 2)
- 66.7%, 100%, and 150% (intervals of 1.5).

The appropriateness of the choice of the suitable log-dose interval will depend on the antibiotic being tested and its limits of acceptance. In some countries more than three different standard disc contents are used.

The standard discs shall be dried rapidly in circulating air, in a vacuum, or in a desiccator. If they are stored before use such storage shall be in a desiccator under refrigeration for a period of time not exceeding that for which adequate stability data are available.

¹ Guidance to national control authorities on the choice of a suitable paper can be provided by Biologics, World Health Organization, 1211 Geneva 27, Switzerland.
5.2.2 Assay media

A medium that has been shown to be satisfactory for the assay of the appropriate antibiotic shall be used.

Suitable media are described in the International Pharmacopeia,\textsuperscript{1} national pharmacopoeias, and the Regulations of the Food and Drug Administration of the USA.\textsuperscript{2}

5.2.3 Assay organisms

A microorganism which has been found satisfactory for the assay of the appropriate antibiotic shall be used.

Suitable assay organisms are described in the International Pharmacopeia,\textsuperscript{1} national pharmacopoeias, and the Regulations of the Food and Drug Administration of the USA.\textsuperscript{2}

5.2.4 Procedure

The assay shall be carried out using flat-bottomed Petri dishes or large square plates containing a layer of seeded assay medium approximately 4 mm in depth. Replica standard discs containing the three different contents and replica sample discs are placed on the agar surface in a random arrangement (see Part C, section 2.1.1, for directions for the storage and handling of discs). Such replication shall be either within the same plate or on several such plates. The extent of replication necessary will vary according to the technique used but shall not be less than fivefold. The discs are placed in position, using forceps, within as short a period as possible, and gently pressed down to ensure a uniform contact with the agar surface. The plates are incubated overnight at the temperature appropriate for the particular microorganism. After incubation, the diameters of the zones of inhibition are measured as accurately as possible. A log-dose/response line is constructed using the means of the responses from all plates to the


\textsuperscript{2} Ibid., Section 460.6(b).
three different standard disc contents. The content of each sample disc is determined from this line.

More details of assay procedures for antibiotics are to be found in the International Pharmacopoeia, national pharmacopoeias, and the Regulations of the Food and Drug Administration of the USA. A suggested method of calculating the content from the assay data is given in Appendix 1.

5.2.5 Limits of acceptance

The content is satisfactory if the mean content of the sample discs is found to be within 75–135% of the labelled amount.

National control authorities should specify criteria for uniformity of content. A suitable measure of uniformity used in one country requires that the range of differences of zone sizes obtained in assaying replicate sample discs should not exceed 2.5 mm for 90% of the discs tested.

5.3 Sterility

Discs are not expected or required to be sterile but should be free from contamination that would interfere with their normal use, e.g., microorganisms likely to cause inactivation of the antibiotic or to obscure the zone of inhibition.

6. RECORDS

In addition to "Good Practices in the Manufacture and Quality Control of Drugs" the requirements given in Part A, section 6.1, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) shall apply.

7. SAMPLES

Samples from each batch shall be taken in sufficient amount to satisfy the requirements for samples of the national control laboratory.

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2 United States of America. Code of Federal Regulations, Title 21, Chapter I, Part 460, subpart A, section 460.8(c) to 460.8(e) (1).
Additional samples shall be retained throughout the dating period as reference material.

8. LABELLING

All products shall be clearly identified by labels. The information given on the label on the container or the label on the package shall be determined by the national control authority.

The label printed on or affixed to each container shall show at least:

- the name of the product (i.e., the international name and/or the proper name);
- the number of discs in the container;
- the nominal content of the antibiotic in each disc;
- the name and address of the manufacturer;
- the batch number;
- the expiry date;
- the conditions of storage.

The leaflet accompanying the container shall describe directions for the use of the discs, having regard to the requirements in Part C.

Each disc shall be marked with a code which identifies the antibiotic contained in it (see Part A, section 1.6).

PART B:

NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for the quality control system given in section 10 of "Good Practices in the Manufacture and Quality Control of Drugs"\(^1\) shall apply.

The national control authority shall specify the content of antibiotics for antibiotic susceptibility discs (see Part A, section 1.5).

The detailed test procedures for production and control shall be those approved by the national control authority. The national control authority shall provide or approve the antibiotic reference preparations for preparing standard discs or for the standardization of laboratory reference preparations for preparing standard discs.


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The national control authority shall approve the recommended directions for use of the discs including the zone diameter interpretative criteria to be used in reporting clinical isolates as either susceptible, intermediate, or resistant.

Criteria approved in one country are given in Appendix 2. Other categories of interpretation may be approved and the following is an example:  

*Group 1*: Includes high degrees of bacterial susceptibility that make *in vivo* response probable when mild to moderately severe systemic infections are treated with usual dosage of antibiotic. Group 1 can be designated as susceptible without further qualification.

*Group 2*: Includes degrees of susceptibility which make *in vivo* response probable in systemic infections when the antibiotic is given in high dosage or up to the limits of toxicity.

*Group 3*: Includes degrees of susceptibility which make *in vivo* response probable only in the treatment of localized infections at sites where the antibiotic can be concentrated by physiological processes or local application.

*Group 4*: Includes organisms of a degree of resistance which make *in vivo* response improbable. This group can be designated as resistant without further qualification.

The national control authority should seek clinical advice before approving interpretative criteria.

The national control authority shall approve the performance test and provide or approve the reference strains to be used in determining the adequacy of the procedure for testing the susceptibility of microorganisms to antibiotics as performed in particular laboratories. The national control authority shall approve the acceptable ranges of zone diameters to be obtained when each antibiotic susceptibility disc is tested for performance against the reference strains (see Part C, section 4).

2. RELEASE AND CERTIFICATION

A batch of antibiotic susceptibility discs shall be released only if it fulfills Part A of the present requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify whether or not the batch of susceptibility

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discs in question meets all national requirements as well as Part A of the present requirements. The certificate shall further state the results of the assay for content and uniformity and the batch 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 antibiotic susceptibility discs between countries.

**PART C:**

**DIRECTIONS FOR USE OF ANTIBIOTIC SUSCEPTIBILITY DISCS**

In the commonly used agar diffusion method the antibiotic diffuses from a disc placed on a solid agar medium, the surface of which has been inoculated with the microorganism under investigation. The presence and size of a zone around the disc within which growth is inhibited is an indication of the susceptibility of the microorganism to the antibiotic. The WHO Expert Committee on Antibiotics recommended the broad principles to be followed in order to obtain satisfactory results in susceptibility testing. The Committee noted that the results of any susceptibility measurements do not yield absolute values because they are influenced, sometimes markedly, by the test conditions used. Differences in such factors as the size of the inoculum, the composition of the medium, pH, atmosphere, and incubation temperature may all affect the amount of antibiotic required to inhibit the organism *in vitro*. Thus the minimum inhibitory concentration of an antibiotic for an organism will depend on the conditions of the test.

It has not been possible to provide directions for use of antibiotic susceptibility discs that are internationally acceptable in all details. Nevertheless guidelines are given that should reduce variability in results between laboratories.

Some details of one procedure accepted on a national basis are included as an example of a method that is being used in more than one country. Full details of the method have been published.²

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² UNITED STATES OF AMERICA. Code of Federal Regulations, Title 21, Chapter I, Part 460, subpart A, section 460.1(c) (3).
1. TERMINOLOGY

Clinical isolate. A microorganism isolated from a clinical specimen to be tested for its susceptibility to antibiotics.

Reference strains. Stock culture(s) to be used in clinical laboratories to check the performance of the method of determining the susceptibility of microorganisms to antibiotics. Reference strains have well defined characteristics and stable behaviour in the susceptibility testing procedure. They are also intended for use in controlling new batches of agar medium as required in Part D and changes in methods and materials used in manufacturing such media.

Performance test. Procedure for determining the validity of antibiotic susceptibility disc tests performed in a given clinical laboratory, reference strains being used to verify that zones of inhibition within expected ranges of sizes are obtained.

Minimum inhibitory concentration (MIC). The minimum concentration of an antibiotic giving complete inhibition of growth of a particular microorganism as judged by the naked eye after a given period of incubation.

Regression line. The linear relationship (line of best fit) shown to exist between the logarithm of the MIC and the diameter of the zone of inhibition produced by a fixed disc content of a given antibiotic for microorganisms of comparable growth rate but of different antibiotic susceptibilities. Such a line shall be determined experimentally using many strains of microorganisms.

A minimum of 100 strains of recently isolated and commonly encountered pathogens has generally been used and twice tested on two occasions in each of two or more laboratories. The strains should be selected to cover the range of clinically important susceptibilities, and if possible there should be at least 10 strains with MICs at each dilution increment.

At least 80 of the strains should have growth rates not less than that of enterococci and extending up to that of the more rapidly growing Enterobacteriaceae. A minimum of four genera should be included and all strains should be characterized. A separate regression line should be determined for a fixed disc content of each antibiotic.

Examples of regression lines obtained by different laboratories, indicating the variations that may occur between laboratories have been described.1 Details are given of the preparation of

such lines, the precautions that must be taken in preparing them and the different sources of error. At present it is not possible to define regression lines that will be universally applicable in all laboratories, although this might be possible in the future with the aid of reference microorganisms of defined susceptibility.

2. REAGENTS

2.1 Discs

Antibiotic susceptibility discs complying with Parts A and B of these requirements shall be used.

2.1.1 Storage of discs

When received, discs shall be stored in their containers at between 
$-20^\circ C$ and $8^\circ C$. When needed, one container is removed from storage and before being opened allowed to stand at room temperature long enough to reach ambient temperature in order to prevent condensation of water vapour on the discs. After discs have been removed, the container shall be closed again and stored at between 
$-20^\circ C$ and $8^\circ C$.

For discs containing certain antibiotics (e.g., penicillins and cephalosporins) the period of storage after opening the container should not be more than one week.

2.2 Agar medium

Agar medium that complies with Part D of these requirements shall be used.

Mueller-Hinton agar medium has been found to be suitable and it is available commercially in a dehydrated form.

2.2.1 pH

The pH of the agar medium shall be 7.2 to 7.4 and, if necessary, shall be adjusted using sodium hydroxide or hydrochloric acid.

The exact method by which the pH is checked will depend upon the type of equipment available in a given laboratory. After the medium has cooled to room temperature its pH is checked after macerating a small amount with an equal quantity of freshly distilled water. Alternative methods are to allow a small amount of the medium to solidify around a pH electrode in a small beaker or pH cup or to use a properly calibrated surface or probe electrode.
2.3 Liquid medium

A suitable liquid medium for preparing the inoculum shall be used.
Soybean-casein digest medium\(^1\) and Mueller-Hinton broth are suitable.

2.4 Turbidity standard

A suitable turbidity standard may be prepared by mixing solutions of 0.048 mol/l BaCl\(_2\) and 0.18 mol/l H\(_2\)SO\(_4\). If inoculation is performed by swabbing, a turbidity standard prepared using 0.5 ml and 99.5 ml, respectively, of these solutions is suitable.

2.5 Petri dishes

Either glass or plastic dishes with a flat internal bottom surface shall be used.
Sterile dishes are commonly used.

3. PROCEDURES

3.1 Preparation of plates

The medium shall be poured into dishes on a flat horizontal surface to a uniform depth of 4 mm. This corresponds to 60 ml of medium for dishes of 14 cm internal diameter and 25 ml for dishes of 9 cm internal diameter.

Freshly prepared plates of solidified medium may be stored at 4°C and protected from desiccation.
Plates should normally be used within seven days, but under certain conditions they may be kept for a longer period.

Immediately before inoculation the plate shall be at room temperature and the surface of the solid medium shall be allowed to dry if necessary.

3.2 Preparation of inoculum for seeding by swabbing or flooding

Normally the inoculum shall be derived from a plate seeded with the original clinical isolate. Whenever possible, portions of 10 colonies of the organism to be tested shall be suspended in liquid medium. The

density of the inoculum shall be carefully adjusted to yield a confluent
growth after incubation of the plate, as described in Part C, section 3.6.

The inoculum may require a period of growth in liquid medium
for several hours, possibly overnight. In some laboratories the
inoculum is adjusted using a suitable turbidity standard to meet
the conditions described above. In cases of clinical urgency the
original clinical specimen (for instance cerebrospinal fluid) may
be used as an inoculum. Preliminary and rapid results may thus
be obtained if the resulting culture is pure and the density of
growth on the susceptibility plate is appropriate.

3.3 Preparation of inoculum for seeding of plates by agar overlay

A suspension of just visible turbidity is prepared in 0.5 ml of liquid
medium using where possible parts of 10 colonies. The suspension is
incubated at 35-37°C for at least 4 hours but not more than 8 hours
and is used to inoculate melted agar at a temperature of 45-50°C.
One microlitre is used to inoculate 9.0 ml of the agar. Inoculum and
agar are then mixed by gentle inversion and used immediately.

A calibrated loop may by used to measure the inoculum.

3.4 Seeding of plates

The plate shall be inoculated evenly over the entire surface of the
solid medium to give the required density of confluent growth.

The density of the inoculum may be defined accurately by the
count of colonies obtained per square centimetre.

One of the methods described in the following three paragraphs
shall be used.

3.4.1 Swabbing

The inoculum shall be distributed evenly by careful streaking in
three directions with a cotton swab.

A bent glass rod may also be used in order to obtain even
distribution. The method of inoculation may be varied but
must be such that it gives uniform seeding of the plate and the
density of growth required. The surface of the medium is
allowed to dry for up to 15 minutes with the Petri dish lid
tipped.
3.4.2 Flooding

The required density of confluent growth can also be obtained by a flooding technique, using 3-5 ml of the appropriate dilution of the suspension of the organism to inoculate a 14 cm plate, or 2 ml to inoculate a 9 cm plate. The inoculum is pipetted onto the plate and distributed evenly over the surface of the medium by gently rocking or tilting the plate. The plate is then tipped, the excess fluid is allowed to drain and is removed with a pipette. The surface of the medium is allowed to dry for up to 15 minutes with the Petri dish lid tipped.

3.4.3 Agar overlay

The inoculum shall be spread over the surface of a Petri dish or plate containing a 4 mm depth of agar medium. The volume of the overlay shall be sufficient to produce a uniform layer 0.6 mm thick (i.e., 9 ml for plates of 14 cm internal diameter or 3.75 ml for plates of 9 cm internal diameter). The surface of the medium is allowed to dry for up to 15 minutes with the Petri dish lid tipped.

3.5 Application of discs

The antibiotic discs shall be applied to the surface of the seeded and dried plate and lightly pressed down immediately. Discs shall be separated by a sufficient distance to reduce the likelihood of serious overlapping of zones and shall be at least 1.5 cm from the edge of the plate.

3.6 Incubation

The plate shall be incubated overnight (about 18 hours) at 35-37°C in an inverted position.

Incubation is normally aerobic, but anaerobic or CO₂ incubation may be necessary for some species.

If anaerobic or CO₂ incubation is used it is important to recognize that regression lines or other interpretative criteria developed from data obtained using aerobic conditions should not be used unless they have been shown experimentally to be applicable.

1 UNITED STATES OF AMERICA. Code of Federal Regulations, Title 21, Chapter I, Part 460, subpart A, section 460.1(c) (2).
3.7 Reading of zone diameters

The diameter of each zone of inhibition shall be measured with sufficient accuracy to determine the proper category of susceptibility (see Appendix 2).

Some laboratories measure each zone of inhibition to the nearest whole millimetre, using templates.

When reading the zones of inhibition in the performance test (see Part C, section 4) the zone diameters shall be measured to the nearest whole millimetre.

Very small colonies within the zone seen under certain conditions of illumination should be ignored. Certain antibiotics may produce poorly defined zone edges. In such cases only the clear zone of inhibition should be measured and any barely visible colonies should be ignored.

Certain organisms may produce large colonies just inside the periphery of the inhibition zone given by certain antibiotics, e.g., ampicillin and cephaloridine. These colonies are taken to define the edge of the zone. If colonies occur throughout the zone of inhibition, the culture should be checked for purity and the test repeated. If the cell culture is found to be pure and such colonies are still present they shall be regarded as significant, calling for special interpretation or investigation.

Strains of Proteus may swarm into zones of inhibition produced by certain antibiotics (e.g., chloramphenicol). The zones are usually clearly outlined under the spreading organism and the veil of swarming should be ignored. If difficulty in measuring is encountered, it usually indicates that an excessively heavy inoculum has been used.

3.8 Interpretation of results

There has been no international agreement on the basis of categorization of susceptibility.

One set of criteria for interpretation, based on the Bauer-Kirby procedure 2 is published in the Regulations of the United States Food and Drug Administration. 3 This is reproduced in Appendix 2 as one example of an interpretative procedure that has been found to be satisfactory using the techniques and materials described in these requirements.

3 UNITED STATES OF AMERICA. Code of Federal Regulations, Title 21, Chapter I, Part 460, subpart A, section 460.1(c) (2) (B).
3.8.1 Limitations of the disc diffusion method

There are certain inherent limitations to diffusion tests. Criteria of evaluation derived from studies on rapidly growing microorganisms are not applicable to slow-growing ones unless they have been shown experimentally to be applicable; otherwise such slow-growing organisms shall be tested by dilution methods. In addition, disc tests with the poorly diffusing polypeptide antibiotics—colistin and polymyxin B—are unreliable in detecting susceptibility; therefore dilution methods shall be employed whenever these antibiotics are considered for use in treating systemic infections.

3.8.2 Problems of penicillin-resistant and meticillin-resistant staphylococci

Penicillinase-producing staphylococci are readily detected by diffusion tests. They yield smaller zone sizes with benzylpenicillin and ampicillin than do non-penicillinase-producing strains and also produce sharply defined zone edges in which colonies at the edge of the zone are the same size or larger than those on other parts of the plate. This is in contrast to the diffuse edge encountered with non-penicillinase producers.

The ability of diffusion methods to detect strains of staphylococci that are heteroresistant to the penicillinase-stable penicillins and to the cephalosporins is dependent on the use of a sufficiently heavy inoculum and is influenced by the medium, the antibiotic used, and the incubation time and temperature. Special tests shall be performed at an incubation temperature of 30°C or on a culture medium containing 5% sodium chloride; discs containing a penicillinase-resistant penicillin such as meticillin or oxacillin are used for such testing.

4. PERFORMANCE TEST

To check the validity of the antibiotic susceptibility test procedure, antibiotic susceptibility discs shall be tested against reference strains of microorganisms on a daily basis.

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4.1 Reference strains

The reference strains used shall be approved by the national control authority.

The following have been used:
- *Staphylococcus aureus* (ATCC 25923; NCTC 6571)
- *Escherichia coli* (ATCC 25922; NCTC 10418)
- *Pseudomonas aeruginosa* (ATCC 27853; NCTC 10662)

These strains can be obtained from the American Type Culture Collection,¹ and the National Collection of Type Cultures.²

4.2 Procedure

Inoculated plates of the reference strains shall be prepared using the procedure described in Part C, sections 3.1 to 3.4. Each reference strain shall be tested against at least one disc of each relevant antibiotic using the procedure described in Part C, sections 3.5 to 3.7.

4.3 Evaluation of performance

The zone size obtained with each antibiotic susceptibility disc shall be compared with the range of zone sizes approved by the national control authority as those to be expected for the appropriate organism (see Part B, section 1).

The following ranges have been found appropriate for antibiotic susceptibility discs 6.35 mm in diameter.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Minimum Zone Diameter (mm)</th>
<th>Maximum Zone Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>amikacin</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>ampicillin</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>carbenicillin</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td>cefalotin</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>colistin</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>erythromycin</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>gentamicin</td>
<td>19</td>
<td>26</td>
</tr>
<tr>
<td>kanamycin</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>neomycin</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>polymyxin B</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>streptomycin</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>tetracycline</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>tobramycin</td>
<td>18</td>
<td>26</td>
</tr>
</tbody>
</table>

¹ American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA.
Staphylococcus aureus (ATCC 25923)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Disc content</th>
<th>Expected zone diameters (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>amikacin</td>
<td>10 µg</td>
<td>18–24</td>
</tr>
<tr>
<td>ampicillin</td>
<td>10 µg</td>
<td>24–35</td>
</tr>
<tr>
<td>bacitracin</td>
<td>10 IU</td>
<td>17–22</td>
</tr>
<tr>
<td>benzylpenicillin</td>
<td>6 µg</td>
<td>26–37</td>
</tr>
<tr>
<td>cefalotin</td>
<td>30 IU</td>
<td>23–37</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>30 µg</td>
<td>19–26</td>
</tr>
<tr>
<td>clindamycin</td>
<td>2 IU</td>
<td>23–29</td>
</tr>
<tr>
<td>erythromycin</td>
<td>15 IU</td>
<td>22–30</td>
</tr>
<tr>
<td>gentamicin</td>
<td>10 IU</td>
<td>19–27</td>
</tr>
<tr>
<td>kanamycin</td>
<td>30 IU</td>
<td>19–26</td>
</tr>
<tr>
<td>meticillin</td>
<td>5 µg</td>
<td>17–22</td>
</tr>
<tr>
<td>neomycin</td>
<td>30 IU</td>
<td>18–26</td>
</tr>
<tr>
<td>novobiocin</td>
<td>30 IU</td>
<td>22–31</td>
</tr>
<tr>
<td>oleandomycin</td>
<td>15 IU</td>
<td>19–28</td>
</tr>
<tr>
<td>polymyxin B</td>
<td>300 IU</td>
<td>7–13</td>
</tr>
<tr>
<td>streptomycin</td>
<td>10 IU</td>
<td>14–22</td>
</tr>
<tr>
<td>tetracycline</td>
<td>30 IU</td>
<td>19–28</td>
</tr>
<tr>
<td>tobramycin</td>
<td>10 µg</td>
<td>19–29</td>
</tr>
<tr>
<td>vancomycin</td>
<td>30 IU</td>
<td>15–19</td>
</tr>
</tbody>
</table>

Pseudomonas aeruginosa (ATCC 27853)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Disc content</th>
<th>Expected zone diameters (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>amikacin</td>
<td>10 µg</td>
<td>15–22</td>
</tr>
<tr>
<td>carbenicillin</td>
<td>100 µg</td>
<td>20–24</td>
</tr>
<tr>
<td>gentamicin</td>
<td>10 IU</td>
<td>16–21</td>
</tr>
<tr>
<td>tobramycin</td>
<td>10 µg</td>
<td>19–25</td>
</tr>
</tbody>
</table>

All zone diameters shall be within the expected range.

PART D:

Requirements for agar medium for antibiotic susceptibility testing using antibiotic susceptibility discs

1. General

In designating a suitable medium for performing antibiotic susceptibility tests using antibiotic susceptibility discs by the agar diffusion technique, the following characteristics are considered desirable.
(1) The composition of the medium should be defined, at least to the point of specific production details, for crude components such as peptone and agar. Ideally the medium should be fully synthetic, but no such medium is generally available.

(2) Susceptibility test results with the appropriate reference strains should be satisfactory.

(3) The medium, without enrichment, should support good growth of the majority of rapidly growing pathogens for which susceptibility tests are required.

(4) The medium should not be antagonistic to any of the antibiotics with which susceptibility tests are made.

(5) The medium should resist marked pH change during the growth of common pathogenic species.

(6) The medium should be approximately isotonic to blood and suitable for the addition of blood, which is necessary for the growth of fastidious organisms.

(7) The medium should be reproducible so that batches from different sources have similar performance and could be available generally.

Mueller-Hinton agar medium from different sources has been found to meet most of these criteria. A large amount of information has been collected on tests performed using this medium. It has shown good batch-to-batch reproducibility in disc diffusion antibiotic susceptibility tests, it is low in sulfonamide and tetracycline inhibitors, and it gives satisfactory growth of most pathogens. With the addition of 5% defibrinated blood (free from antimicrobial activity) Mueller-Hinton agar can be used in performing antibiotic susceptibility tests on certain fastidious organisms that cannot grow on the medium alone.

Different media have been proposed with "physiological" concentrations of various ions and with nitrogenous components different from those of Mueller-Hinton agar. The interpretation of zone sizes (see Part C, section 3.8) based on Mueller-Hinton agar may not be applicable to such different media, nor can it be assumed that regression lines determined using Mueller-Hinton agar can be used for such different media.

2. FORMULA

Mueller-Hinton agar medium is based on a formula for a liquid medium \(^1\) with agar added:

dehydration infusion from 300 g beef
acetic digest of casein .................................. 17.5 g
corn starch .............................................. 1.5 g
agar ......................................................... 17.0 g
distilled water to make ................................ 1000 ml
pH after autoclaving ...................................... 7.4

Mueller-Hinton agar medium is available in a dehydrated form, so that when it is reconstituted with distilled water to make 1000 ml and autoclaved it has a pH of 7.4.

The user should check the pH and adjust it if necessary (see Part C, section 2.2.1).

Because of the influence of Mg$^{2+}$ and Ca$^{2+}$ on the susceptibility of *Pseudomonas aeruginosa* to aminoglycoside antibiotics, it has been recommended that Mueller-Hinton agar contain 20–35 mg of Mg$^{2+}$ and 50–100 mg of Ca$^{2+}$ per litre. At this level of free divalent cations, which are known to chelate tetracyclines, the zone sizes obtained with tetracycline discs are slightly reduced in relation to the zone sizes obtained using media of lower content of Mg$^{2+}$ and Ca$^{2+}$.

Unless a laboratory is experienced in making media it is advised to obtain dehydrated agar medium from a batch that has been shown to be suitable.

3. PERFORMANCE TESTING

Batches of agar medium should be controlled using the performance test described in Part C, section 4. The mean zone size for each antibiotic (obtained from at least four determinations on separate plates) shall be within the expected range of zone diameters listed in Part C, section 4.3.

Appendix 1

CALCULATION OF DISC CONTENT FROM ASSAY DATA

Determine the logarithm ($x$) of each dose of standard and the mean response ($y$) to these doses. Using the three values of $x$ and the three corresponding values of $y$, calculate $\Sigma x$, $\Sigma x^2$, $(\Sigma xy)^2$, $\Sigma y$, and $\Sigma xy$. 

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Calculate the slope \( b \), which is the regression coefficient and the \( y \)-intercept \( a \) of the standard response line by the following equations:

\[
b = \frac{n \sum xy - (\sum x)(\sum y)}{n \sum x^2 - (\sum x)^2} \quad \text{and} \quad a = \frac{\Sigma y - b \Sigma x}{n}
\]

where \( n \) = the number of standard doses.

Determine the response \( (Y) \) for each sample disc. Using the regression equation, calculate the content \( (X) \) for any observed response of sample disc as follows:

\[
X = \text{antilog} \left[ \frac{Y - a}{b} \right]
\]

The following example illustrates the mathematical calculation of the content of a sample disc:

<table>
<thead>
<tr>
<th>Standard doses (μg or IU per disc)</th>
<th>15.0</th>
<th>30.0</th>
<th>60.0</th>
<th>( n = 3 )</th>
</tr>
</thead>
</table>

Log doses \( (x) \)

<table>
<thead>
<tr>
<th>( x )</th>
<th>1.17609</th>
<th>1.47712</th>
<th>1.77815</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Sigma x )</td>
<td>4.43136</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( (\Sigma x)^2 )</td>
<td>19.63698</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( x^2 \)

<table>
<thead>
<tr>
<th>( x^2 )</th>
<th>1.38319</th>
<th>2.18189</th>
<th>3.16182</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Sigma x^2 )</td>
<td>6.72690</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Standard zones (mm)

<table>
<thead>
<tr>
<th>Standard zones (mm)</th>
<th>16.6</th>
<th>18.2</th>
<th>19.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.0</td>
<td>18.9</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>17.0</td>
<td>18.9</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td>17.0</td>
<td>19.1</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>16.9</td>
<td>18.6</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td>16.3</td>
<td>18.2</td>
<td>18.7</td>
<td></td>
</tr>
</tbody>
</table>

Mean responses \( (y) \)

<table>
<thead>
<tr>
<th>Mean responses ( (y) )</th>
<th>16.8</th>
<th>18.7</th>
<th>19.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Sigma y )</td>
<td>55.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( xy \)

<table>
<thead>
<tr>
<th>( xy )</th>
<th>19.75833</th>
<th>27.62217</th>
<th>35.20739</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Sigma xy )</td>
<td>82.58790</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[
b = \frac{3(82.58790) - (4.43136)(55.3)}{3(6.72690) - (19.63698)} = 4.98
\]

\[
a = \frac{55.3 - (4.98)(4.43136)}{3} = 11.07728
\]

Response, \( Y \), of sample disc = 18.9 mm

Calculated content, \( X \), of sample disc = antilog \( \left[ \frac{18.9 - 11.07728}{4.98} \right] = 37 \) µg or IU.

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### Appendix 2

**CRITERIA FOR INTERPRETATION BASED ON THE BAUER-KIRBY PROCEDURE**

(disc diameter 6.35 mm)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Disc content</th>
<th>Diameter (mm) of zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
</tr>
<tr>
<td>amikacin</td>
<td>10 µg</td>
<td>11 or less</td>
</tr>
<tr>
<td>ampicillin 1 when testing grama-negative microorganisms and enterococi</td>
<td>10 µg</td>
<td>11 or less</td>
</tr>
<tr>
<td>ampicillin 1 when testing staphylococci and benzylpenicillin-susceptible microorganisms</td>
<td>10 µg</td>
<td>20 or less</td>
</tr>
<tr>
<td>ampicillin 1 when testing <em>Haemophilus</em> species</td>
<td>10 µg</td>
<td>19 or less</td>
</tr>
<tr>
<td>bacitracin</td>
<td>10 IU</td>
<td>8 or less</td>
</tr>
<tr>
<td>benzylpenicillin when testing staphylococci 8</td>
<td>6 µg</td>
<td>20 or less</td>
</tr>
<tr>
<td>benzylpenicillin when testing other microorganisms 8</td>
<td>6 µg</td>
<td>11 or less</td>
</tr>
<tr>
<td>carbencillin when testing Proteus species and <em>Escherichia coli</em></td>
<td>100 µg</td>
<td>17 or less</td>
</tr>
<tr>
<td>carbencillin when testing <em>Pseudomonas aeruginosa</em></td>
<td>100 µg</td>
<td>13 or less</td>
</tr>
<tr>
<td>cefalotin when reporting susceptibility to cefalotin, cefaloridine, cefalexin, cefapirin, cefazolin, cefacetrile, and ceftadine</td>
<td>30 IU</td>
<td>14 or less</td>
</tr>
<tr>
<td>cefalotin when reporting susceptibility to cefalotin and cefamandole</td>
<td>30 IU</td>
<td>14 or less</td>
</tr>
<tr>
<td>clindamycin 8 when reporting susceptibility to clindamycin</td>
<td>2 IU</td>
<td>16 or less</td>
</tr>
<tr>
<td>clindamycin when reporting susceptibility to lincomycin</td>
<td>30 IU</td>
<td>8 or less</td>
</tr>
<tr>
<td>colistin</td>
<td>15 IU</td>
<td>13 or less</td>
</tr>
<tr>
<td>erythromycin</td>
<td>10 IU</td>
<td>12 or less</td>
</tr>
<tr>
<td>gentamicin</td>
<td>30 IU</td>
<td>13 or less</td>
</tr>
<tr>
<td>kanamycin</td>
<td>5 µg</td>
<td>9 or less</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Disc content</td>
<td>Resistant</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>neomycin</td>
<td>30 IU</td>
<td>12 or less</td>
</tr>
<tr>
<td>novobiocin</td>
<td>30 IU</td>
<td>17 or less</td>
</tr>
<tr>
<td>oleandomycin §</td>
<td>15 IU</td>
<td>11 or less</td>
</tr>
<tr>
<td>polymyxin B</td>
<td>300 IU</td>
<td>8 or less</td>
</tr>
<tr>
<td>rifampicin when testing Neisseria meningitidis</td>
<td>5 µg</td>
<td>24 or less</td>
</tr>
<tr>
<td>streptomycin</td>
<td>10 IU</td>
<td>11 or less</td>
</tr>
<tr>
<td>tetracycline §</td>
<td>30 IU</td>
<td>14 or less</td>
</tr>
<tr>
<td>tebramycin</td>
<td>10 µg</td>
<td>11 or less</td>
</tr>
<tr>
<td>vancomycin</td>
<td>30 IU</td>
<td>9 or less</td>
</tr>
</tbody>
</table>

1 The ampicillin disc is used for testing susceptibility to ampicillin, amoxicillin, and beta-lactam. When testing *Haemophilus* species, an isolate exhibiting ampicillin susceptibility and benzylpenicillin resistance should be considered resistant to ampicillin.
2 The 30 IU cefalotin disc cannot be relied upon to detect resistance of staphylococci to cephalosporin class antibiotics. Staphylococci exhibiting resistance to meticillin should be reported as resistant to cephalosporin class antibiotics.
3 The clindamycin disc is used for testing susceptibility to both clindamycin and lincomycin.
4 Colistin and polymyxin B diffuse poorly in agar, and the accuracy of the diffusion method is thus less than is the case with other antibiotics. Resistance is always significant, but when treatment of systemic infections due to susceptible strains is considered it is wise to confirm the results of a diffusion test with a dilution method.
5 The meticillin disc is used for testing susceptibility to all penicillinase-resistant penicillins—i.e., meticillin, cloxacillin, dicloxacillin, oxacillin and nafcillin—and for verifying resistance to cephalosporin class antibiotics (see Part C, section 3.8.2).
6 Not applicable to medium that contains blood.
7 The oleandomycin disc is used for testing susceptibility to oleandomycin and troleandomycin.
8 The benzylpenicillin disc is used for testing susceptibility to all penicillinase-susceptible penicillins except ampicillin and carbenicillin—i.e., benzylpenicillin, phenoxymethylpenicillin, and phenicillin.
9 This category includes some organisms such as enterococci and gram-negative bacilli that may cause systemic infections treatable with high doses of benzylpenicillin. Such organisms should only be reported susceptible to benzylpenicillin and not to phenoxymethylpenicillin or phenicillin.
10 The tetracycline disc is used for testing susceptibility to all tetracyclines—i.e., chlorotetracycline, demeclocycline, doxycycline, metacycline, oxytetracycline, rolitetracycline, minocycline, and tetracycline. Some organisms that are resistant to tetracycline may be susceptible to minocycline.
Annex 6

REQUIREMENTS FOR BIOLOGICAL SUBSTANCES
AND OTHER SETS OF RECOMMENDATIONS

The specification of requirements to be fulfilled by preparations of biological substances is necessary in order to ensure that these products are safe, reliable, and potent prophylactic or therapeutic agents. International recommendations on requirements are intended to facilitate the exchange of biological substances between different countries and to provide guidance to workers responsible for the production of these substances as well as to others who may have to decide upon appropriate methods of assay and control.

Recommended requirements and sets of recommendations concerned with biological substances formulated by international groups of experts and published in the WHO Technical Report Series are listed hereunder:

<table>
<thead>
<tr>
<th>No.</th>
<th>Year</th>
<th>Requirements for Biological Substances:</th>
</tr>
</thead>
<tbody>
<tr>
<td>178</td>
<td>1959</td>
<td>1. General Requirements for Manufacturing Establishments and Control Laboratories¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Requirements for Poliomyelitis Vaccine (Inactivated)¹</td>
</tr>
<tr>
<td>179</td>
<td>1959</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Requirements for Yellow Fever Vaccine¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Requirements for Cholera Vaccine¹</td>
</tr>
<tr>
<td>180</td>
<td>1959</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5. Requirements for Smallpox Vaccine¹</td>
</tr>
<tr>
<td>200</td>
<td>1960</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6. General Requirements for the Sterility of Biological Substances¹</td>
</tr>
<tr>
<td>237</td>
<td>1962</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7. Requirements for Poliomyelitis Vaccine (Oral)¹</td>
</tr>
<tr>
<td>274</td>
<td>1964</td>
<td>WHO Expert Committee on Biological Standardization:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8. Requirements for Pertussis Vaccine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate¹</td>
</tr>
<tr>
<td>293</td>
<td>1964</td>
<td>WHO Expert Committee on Biological Standardization:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10. Requirements for Diphtheria Toxoid and Tetanus Toxoid</td>
</tr>
</tbody>
</table>

¹ Refer to subsequent revised requirements.
WHO Expert Group:
Requirements for Biological Substances (Revised 1965)
1. General Requirements for Manufacturing Establishments and Control Laboratories
2. Requirements for Poliomyelitis Vaccine (Inactivated)
3. Requirements for Poliomyelitis Vaccine (Oral) ¹
4. Requirements for Smallpox Vaccine

WHO Expert Committee on Biological Standardization:
11. Requirements for Dried BCG Vaccine
12. Requirements for Measles Vaccine (Live) and Measles Vaccine (Inactivated)

WHO Expert Committee on Biological Standardization:
13. Requirements for Anthrax Spore Vaccine (Live—for Veterinary Use)
14. Requirements for Human Immunoglobulin
15. Requirements for Typhoid Vaccine
9. Requirements of Procaine Benzylpenicillin in Oil with Aluminium Monostearate (Revisions adopted 1966)

WHO Expert Committee on Biological Standardization:
16. Requirements for Tuberculin
17. Requirements for Inactivated Influenza Vaccine ²

WHO Expert Committee on Biological Standardization:
4. Requirements for Cholera Vaccine (Revised 1968) ³
18. Requirements for Immune Sera of Animal Origin

WHO Expert Committee on Biological Standardization:
19. Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)
20. Requirements for Brucella abortus Strain 19 Vaccine (Live—for Veterinary Use) ³

WHO Expert Committee on Biological Standardization:
Development of a National Control Laboratory for Biological Substances (A guide to the provision of technical facilities)

WHO Expert Committee on Biological Standardization:
21. Requirements for Snake Antivenins

WHO Expert Committee on Biological Standardization:
7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1971)

¹ Refer to subsequent revised requirements.
² Refer to subsequent addendum.
530 1973 WHO Expert Committee on Biological Standardization:
4. Requirements for Cholera Vaccine (Revised 1968) (Addendum 1973)
6. General Requirements for the Sterility of Biological Substances (Revised 1973)
17. Requirements for Inactivated Influenza Vaccine (Addendum 1973)
22. Requirements for Rabies Vaccine for Human Use

565 1975 WHO Expert Committee on Biological Standardization:
Recommendations for the Assessment of Binding Assay Systems (including Immunossay and Receptor Assay Systems) for Human Hormones and their Binding Proteins (A guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytochemical bioassay systems) Development of national assay services for hormones and other substances in community health care

594 1976 WHO Expert Committee on Biological Standardization:
3. Requirements for Yellow Fever Vaccine (Revised 1975)
23. Requirements for Meningococcal Polysaccharide Vaccine¹
20. Specifications of tests used in the Requirements for Brucella abortus Strain 19 Vaccine (Live—for Veterinary Use) (Addendum 1975)

610 1977 WHO Expert Committee on Biological Standardization:
23. Requirements for Meningococcal Polysaccharide Vaccine (Addendum 1976)
24. Requirements for Rubella Vaccine (Live)
25. Requirements for Brucella melitensis Strain Rev. 1 Vaccine (Live—for Veterinary Use)

¹ Refer also to subsequent addendum.
Annex 7

BIOLOGICAL SUBSTANCES: INTERNATIONAL STANDARDS, REFERENCE PREPARATIONS, AND REFERENCE REAGENTS

The list of international biological standards, international biological reference preparations, and international biological reference reagents previously included as annexes to the reports of the WHO Expert Committee on Biological Standardization are issued as a separate publication. The most recent list was published in 1977 and copies may be obtained direct (or through booksellers) from the agents shown on the back cover of this report or they may be ordered from: World Health Organization, Distribution and Sales Service, 1211 Geneva 27, Switzerland.

The Expert Committee, at its twenty-eighth meeting, made the following changes to the lists already published:

**Established**

_Blood Products and Related Substances_

<table>
<thead>
<tr>
<th>Substance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancrod</td>
<td>International Reference Preparation 1976</td>
</tr>
<tr>
<td>Anti-D (anti rh₄) immunoglobulin, human</td>
<td>International Reference Preparation 1976</td>
</tr>
<tr>
<td>Blood coagulation factor IX, human</td>
<td>International Standard 1976</td>
</tr>
<tr>
<td>Plasmin</td>
<td>International Reference Preparation 1976</td>
</tr>
<tr>
<td>Thromboplastin, human, combined</td>
<td>International Reference Preparation 1976</td>
</tr>
</tbody>
</table>

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The above substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England.

Reagents

Adenovirus antisera, equine: International Reference Reagents 1976
Types 4, 19, 20, 22, 23
and 24

These antisera are held and distributed by the Center for Disease Control, Atlanta, GA, 30333, USA. Antisera prepared from the same batch of material as these reference reagents are available in the WHO Virus Reference Centres and are available also on application to the Chief Medical Officer, Virus Diseases, World Health Organization, 1211 Geneva 27, Switzerland.