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Geneva, 27 September–3 October 1983

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GENERAL

The WHO Expert Committee on Biological Standardization met in Geneva from 27 September to 3 October 1983. The meeting was opened on behalf of the Director-General by Dr B. Sankaran, Director, Division of Diagnostic, Therapeutic, and Rehabilitative Technology.

International standards and international reference preparations

In the past, two categories of standard preparations with defined international units of activity have been established, namely, international biological standards and international biological reference preparations. The need for two categories, both serving the same function, had been questioned and was examined by the Committee.

The category of international biological reference preparations was first introduced during a period of rapid development in the antibiotic field. Because the establishment of an international standard, with its defined unit of activity, was a lengthy procedure, it was considered necessary to make internationally accepted reference preparations available as quickly as possible, to avoid the confusion arising from a multiplicity of national units. The stated intention was "to temporarily satisfy a need which, at a later date, could be met by replacement of such international reference preparations by international standards" (WHO Technical Report Series, No. 172, 1959, p. 6). At that period an international reference preparation did not define an international unit of activity. Later, however, the Committee decided that an international unit could be assigned not only to an international standard, but also to an international reference preparation (WHO Technical Report Series, No. 259, 1963, p. 6). This procedure was followed, for example, for the International Reference Preparation of Amphotericin B and the International Reference Preparation of Vancomycin (WHO Technical Report
Series, No. 274, 1964, p. 8). Subsequently, many international reference preparations defining international units of activity were established on the basis of collaborative assays. They have served their intended function satisfactorily but have not been reclassified as international standards.

The Committee, at its present meeting, agreed that it was no longer necessary for international reference preparations with defined units of activity to constitute a separate category, and that all such materials could be considered functionally to be international standards. The question of whether it would be desirable to rename the international biological reference preparations already established as international biological standards was discussed, and it was agreed that to do so would probably cause confusion because of the extensive scientific literature in which their existing names had been used.

The Committee decided that, in the future, a single category of standard preparations would be established for the purpose of defining international units of activity, namely, international biological standards. It would, nevertheless, remain necessary to designate certain materials as international biological reference preparations, but that designation would be restricted to preparations that did not define international units of activity.

The Committee agreed that all international standards and international reference preparations with defined units of activity on which the World Health Assembly had not yet made a recommendation should be presented to the Health Assembly as soon as possible. This procedure was necessary to ensure the continued international acceptance of the various international units of biological activity.

**Paper discs**

In Part A, section 5.2.1 of the *Requirements for antimicrobial susceptibility tests. I. Agar diffusion tests using antimicrobial susceptibility discs* (WHO Technical Report Series, No. 673, 1982, pp. 156–159) it is stated that for *assays of content* of blank discs suitable paper should be used to prepare standard discs; that is, discs impregnated with standard antimicrobial agents. Whether the blank discs to be used routinely by a control laboratory are suitable may be determined by comparing how they perform in the assay system with the performance of blank discs of known suitability.
The Committee was informed that the Food and Drug Administration of the United States of America could no longer supply blank paper discs known to be suitable for the preparation of standard discs. The United States Pharmacopeia was therefore taking steps to obtain a new supply of blank paper discs and to compare their performance with that of the suitable discs previously available, of which small quantities had been recovered from recipients. It was planned to obtain samples from several batches of blank paper discs, and to assess the suitability of each sample by means of preliminary tests. A supply from a batch that had been found suitable would then be obtained and subjected to international collaborative assay against the previously distributed discs.

Rabies vaccine

When the third International Reference Preparation of Rabies Vaccine was established in 1978 (WHO Technical Report Series, No. 638, 1979, p. 15), two candidate preparations of vaccine were compared, by testing their ability to induce antibodies in mice and by a mouse challenge test (NIH test). Supplies of the preparation chosen were limited, however, and work commenced on a replacement preparation.

Since 1978 it has been determined that the content of the immunizing antigen, a glycoprotein, can be measured with a precision of ± 5% by a single radial diffusion (SRD) technique, using specific anti-glycoprotein serum. The potency of the replacement preparation, the International Standard for Rabies Vaccine (see page 15), has been defined on the basis of assays using the SRD technique. Although this technique is not useful for vaccines that contain little glycoprotein, an adjuvant, or any brain tissue or extracts, it is the test of choice for measuring the potency of rabies vaccines produced in tissue culture. Nevertheless, the International Standard for Rabies Vaccine is suitable for use in all potency assay methods.

Tuberculin

It has been estimated that several hundred million people are tested with tuberculin each year; the need for uniformity between tuberculin preparations is obvious. Unfortunately, tuberculin pre-
parations purified in different ways behave differently, so that reaction patterns in different populations may not be comparable. Several approaches have been used in attempting to find a solution to the problem. One has been the establishment of international standard preparations. Another has been to produce large batches of tuberculin preparations and to make them widely available for routine use. Two preparations in particular have been very widely used, one known as PPD-S and the other as RT-23. The first International Standard for Purified Protein Derivative (PPD) of Mammalian Tuberculin was made from PPD-S.

Purified tuberculoproteins are very active substances, and are used in high dilutions. This could result in the adsorption of significant amounts of the tuberculin substances to container surfaces. To minimize adsorption, surface active substances, such as Tween 80, have commonly been added to the diluent. Such additives, however, may influence the activity of different purified tuberculin preparations to varying degrees.

Requirements for Tuberculins were formulated in 1968. It had been realized that the potency of tuberculin preparations could always be expressed in terms of international units, established by means of a biological assay that would be valid for all conditions of use (WHO Technical Report Series, No. 384, 1968, p. 27). The control of potency of tuberculins in the Requirements is, therefore, based on special considerations.

When it was realized that a replacement of the International Standard for Purified Protein Derivative (PPD) of Mammalian Tuberculin was necessary, a preparation was made from the same batch of purified tuberculin (PPD-S) as that used for the first standard and is under investigation. In this way it is hoped to maintain continuity in the activity of the International Unit.

It has been suggested that, since significant developments have taken place in this field in recent years, it would be advisable to study whether tuberculin preparations resulting from the application of such developments would be more suitable than the one at present under investigation, for submission as candidate preparations for the replacement of the International Standard, and whether the Requirements for Tuberculins should be revised. The Committee therefore requested the WHO Secretariat to arrange for consultation with experts in the field.
The potency of diphtheria and tetanus toxoids, adsorbed

The methods currently used to assay the potency of diphtheria and tetanus toxoids require large numbers of animals. However, it may be possible to use fewer animals without changing the principles on which potency control is based. The Committee was informed that an informal WHO consultation of experts would discuss suitable modifications of the Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Revised 1978).

Calibration and expression of results of assays of human gonadotrophins

The standardization of the human gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), is complex. The natural hormones consist of groups of closely similar (but not identical) isohormones, and the molecular forms are believed to differ in pituitary extracts, plasma, and urine.

As the need for international standards suitable for use in bioassays and immunoassays developed, international reference preparations of pituitary and urinary extracts were characterized and established, and international units were assigned for FSH and LH activities according to the principles formulated by the Committee over the years. Moreover, as more highly purified preparations became available they were calibrated and established in replacement of earlier, less pure, reference materials. In the calibration of these international reference materials, efforts were made to make the international units of FSH and LH approximately equivalent in, respectively, the pituitary and urinary extracts, and to ensure that the continuity of the international units in successive replacement preparations was maintained.

The interrelationships of FSH and LH units defined by the various international reference materials are thus complex and have led to confusion. This confusion is reflected in the ways in which certain scientific journals require assay results to be expressed, which, in certain instances, conflict with the Requirements for Immunoassay Kits (WHO Technical Report Series, No. 658, 1981, Annex 8, p. 206) and do not correspond to particular available reference materials. Certain manufacturers thus find themselves obliged to calibrate their assay kits to meet the editorial policies of the journals concerned.
The Committee requested the National Institute for Biological Standards and Control, London, to document for publication the various international gonadotrophin reference materials, in a way similar to the unpublished working document WHO/BS/83.1416, and to bring it to the attention of the editors of the relevant scientific journals. It was hoped that this measure would lead to uniformity in editorial policy with regard to the expression of assay results, in terms of international units of the appropriate current international standard or reference preparation of FSH or LH.

SUBSTANCES

ANTIBIOTICS

1. Amikacin

The Committee noted that analysis of the results of the collaborative assay of the proposed international reference preparation of amikacin, referred to in its thirty-third report (WHO Technical Report Series, No. 687, 1983, p. 16), had been completed (WHO/BS/83.1398).

The Committee established the material studied as the International Standard for Amikacin and defined the activity of the contents of each ampoule of the International Standard for Amikacin as 50 600 International Units of Amikacin.

2. Erythromycin

The Committee noted that, in accordance with the request made in its thirty-third report (WHO Technical Report series, No. 687, 1983, p. 18), the National Institute for Biological Standards and Control, London, had investigated the need for a new international standard for erythromycin (WHO/BS/83.1413).

The Committee agreed that the first and second International Standards for Erythromycin had been shown to be stable and that there was no evidence that the activity defined by the International Unit had changed. Since highly purified preparations of erythromycin might now be available, the Committee requested the WHO
Secretariat to investigate the possibility of establishing an authentic chemical reference substance.

3. *Netilmicin*

The Committee was informed that, in accordance with the request made in its thirty-third report (WHO Technical Report Series, No. 687, 1983, p. 18), the National Institute for Biological Standards and Control, London, had obtained a quantity of netilmicin suitable to serve as an international standard, and that the material would be distributed into ampoules and a collaborative assay arranged.

4. *Sisomicin*

The Committee was informed that the collaborative assay of the preparation of sisomicin, referred to in its thirty-second report (WHO Technical Report Series, No. 673, 1982, p. 17), had been completed and that the results had been analysed.

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

5. *Tobramycin*

The Committee noted that the collaborative assay of the proposed replacement international reference preparation of tobramycin, referred to in its thirty-third report (WHO Technical Report Series, No. 687, 1983, p. 18), was nearing completion (WHO/BS/83.1414).

The Committee authorized the National Institute for Biological Standards and Control, London, to establish this preparation as the International Standard for Tobramycin and, on the basis of the results of the collaborative assay and with the agreement of the participants, to assign the activity in international units of tobramycin to the contents of each ampoule.
ANTIBODIES

6. *Clostridium botulinum* Type B Antitoxin, Equine

The Committee noted that the collaborative assay of the proposed replacement of the International Standard for *Clostridium botulinum* Type B Antitoxin, Equine, referred to in its thirty-third report (WHO Technical Report Series, No. 687, 1983, p. 19), had not been completed, since discrepancies in the results had occurred among the participating laboratories; and that a further collaborative study was planned (WHO/BS/83.1409).

The Committee noted also that since the stock of the international standard was exhausted, the proposed replacement material calibrated against the International Standard for *Clostridium botulinum* Type B Antitoxin, Equine, was being distributed until such time as the proposed replacement standard was established.

7. Monoclonal Antibodies

The Committee noted that the International Association of Biological Standardization had held a meeting on the standardization of monoclonal antibodies, during which a discussion had taken place on the control of such products (WHO/BS/83.1427). The standardization of monoclonal antibodies had been discussed by the Committee at its thirty-third meeting (WHO Technical Report Series, No. 687, 1983, p. 14). It was agreed that the report of the meeting of the International Association of Biological Standardization was timely.

The Committee noted also that methods of producing monoclonal antibodies were undergoing rapid change and would demand the application of new tests. It was agreed that new biological reference materials might be required.

ANTIGENS

8. Lecithin (Bovine Heart)

The Committee noted that the stock of the second International Reference Preparation of Lecithin (Bovine Heart) was exhausted
(WHO/BS/83.1406). The Committee noted also that, in recent years, this international reference preparation had been used so infrequently that replacement was not justified.

The Committee, therefore, discontinued the second International Reference Preparation of Lecithin (Bovine Heart).

9. Rabies Vaccine

The Committee noted that the international collaborative assay on rabies vaccine, referred to in its thirty-third report (WHO Technical Report Series, No. 687, 1983, p. 21), had been completed (WHO/BS/83.1422).

The Committee noted also that closer agreement among laboratories in regard to potency estimates had been obtained with the single radial diffusion (SRD) technique than with either the ELISA or the classical NIH mouse challenge test, but that the SRD technique could be used only for vaccines more potent than the usual vaccines made in animal brains and which did not contain either brain tissue, extracts, or adjuvant.

The Committee established the preparation studied as the International Standard for Rabies Vaccine and assigned a potency of 7.8 International Units of Rabies Vaccine to the contents of each ampoule.

10. Tetanus Toxoid, Adsorbed

The Committee referred to the heterogeneity among different tetanus toxoids that had been demonstrated in the international collaborative study mentioned in its thirty-second report (WHO Technical Report Series, No. 673, 1982, p. 21). The Committee noted that, in a separate study in monkeys, there had been a lack of correlation of antibody responses to toxoids when different vaccine preparations with different potencies expressed in international units had been compared.

The Committee requested the WHO Secretariat to obtain further information on these difficulties, with a view to changing, if necessary, the Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid and Combined Vaccines (Revised 1978) (WHO Technical Report Series, No. 638, 1979, Annex 1, p. 90).
The Committee also requested the State Serum Institute, Copenhagen, to distribute relevant information with ampoules of the second International Standard for Tetanus Toxoid, Adsorbed, so that laboratories calibrating national or working standards would be aware of the difficulties involved in carrying out potency assays of tetanus vaccines.

11. Purified Protein Derivative of Mammalian Tuberculin

The Committee noted that the limited collaborative assay of the proposed replacement preparation for the International Standard for Purified Protein Derivative (PPD) of Mammalian Tuberculin, referred to in its thirty-second report (WHO Technical Report Series, No. 673, 1982, p. 22), had not yet been completed (WHO/BS/83.1408).

The Committee noted also that, since the stocks of the international standard had been exhausted, the proposed replacement preparation, calibrated against the International Standard for Purified Protein Derivative (PPD) of Mammalian Tuberculin, was being distributed until such time as the replacement standard was established.

The Committee requested the WHO Secretariat to convene a meeting of a group of experts to make suggestions on future international standards for purified protein derivative (PPD) of mammalian tuberculin.

12. Short Ragweed Pollen Extract

The Committee noted that, after preliminary investigation of a number of extracts of samples of pollen from the grass, short ragweed (Ambrosia elatior), one preparation had been selected as a proposed international reference material and that an international collaborative study had been completed (WHO/BS/83.1412). The results had shown that, by a number of immunochemical, biochemical, and physicochemical procedures, the activities of the preparation could be differentiated and that a duplicate preparation could be recognized.

The Committee established the proposed reference material as the International Standard for Short Ragweed Pollen Extract and de-
fined the activity of the contents of each ampoule as 100 000 International Units of Short Ragweed Pollen Extract.

13. Timothy Grass Pollen Extract

The Committee noted that, after preliminary investigation of three extracts of samples of pollen from Timothy grass (*Phleum pratense*), one preparation had been selected as a proposed international reference material and that an international collaborative study had been completed (WHO/BS/83.1411). The results had shown that, using several assay systems, all 14 participating laboratories could differentiate the activities of the three preparations included in the study.

The Committee established the proposed reference material as the International Standard for Timothy Grass Pollen Extract and defined the activity of the contents of each ampoule as 100 000 International Units of Timothy Grass Pollen Extract.

**BLOOD PRODUCTS AND RELATED SUBSTANCES**

14. Anti-C Complete and Anti-E Complete Blood Typing Sera

The Committee noted that, in accordance with the request made in its twenty-eighth report (WHO Technical Report Series, No. 610, 1977, p. 10), the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, had obtained materials suitable to serve as international standards for anti-C complete and anti-E complete blood typing sera, and had carried out an international collaborative study (WHO/BS/83.1424).

The Committee authorized the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service to establish the materials studied as the International Standards for Anti-C Complete and Anti-E Complete Blood Typing Sera and, with the agreement of the participants, to assign a unitage to the contents of each ampoule.

The Committee noted also that studies were in progress with a view to establishing an international standard for anti-D complete blood typing serum.
15. **Anti-Varicella Zoster Immunoglobulin**

The Committee was informed that a collaborative study had been organized by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, to assess whether a freeze-dried preparation of anti-varicella zoster immunoglobulin was suitable to serve as an international standard.

16. **Heparin**

The Committee noted that the international collaborative study carried out with a view to replacing the third International Standard for Heparin, referred to in its thirty-third report (WHO Technical Report Series, No. 687, 1983, p. 10), had been completed (WHO/BS/83.1421). The Committee noted also that close agreement in regard to the potency estimates had been obtained for two candidate preparations, when they had been assayed, by a number of methods, against the third International Standard for Heparin.

The Committee established one of the preparations studied as the fourth International Standard for Heparin and, on the basis of the results of the collaborative assay, assigned an activity of 1780 International Units of Heparin to the contents of each ampoule.

17. **Thromboplastin, Bovine, Combined**

The Committee noted that a proposed replacement for the International Reference Preparation of Thromboplastin, Bovine, Combined, had been calibrated in a collaborative study against the International Reference Preparation of Thromboplastin, Human, Combined (WHO/BS/83.1393), in accordance with the Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy (Revised 1982) (WHO Technical Report Series, No. 687, 1983, Annex 3, p. 81). The Committee noted also that the material, coded OBT/79, had been shown to be suitable and that long-term stability studies were being carried out.

The Committee established the proposed material, coded OBT/79, as the second International Reference Preparation of Thromboplastin, Bovine, Combined, and assigned to it an International Sensitivity Index (ISI) of 1.0.
18. Thromboplastin, Human, Plain

The Committee noted the results of the collaborative assay of a proposed international reference preparation of thromboplastin, human, plain, coded BCT/253, calibrated against the International Reference Preparation of Thromboplastin, Human, Combined (WHO/BS/83.1419). The Committee noted also that the preparation had been shown to be suitable and that long-term stability studies were being carried out.

The Committee established the preparation, coded BCT/253, as the second International Reference Preparation of Thromboplastin, Human, Plain, and assigned to it an International Sensitivity Index (ISI) of 1.1.

The Committee discontinued the International Reference Preparation of Thromboplastin, Human, Combined, and agreed that future international reference preparations of thromboplastin should be calibrated against the International Reference Preparation of Thromboplastin, Human, Plain.

ENDOCRINOLOGICAL AND RELATED SUBSTANCES

19. Insulins

The Committee noted that, during the freeze-drying of the purified porcine, bovine, and human insulins mentioned in its thirty-second report (WHO Technical Report Series, No. 673, 1982, p. 29) between 10% and 15% of the material had been chemically modified (WHO/BS/83.1399). Accordingly, the National Institute for Biological Standards and Control, London, had obtained additional quantities of purified porcine, bovine, and human insulin crystals. These materials had been filled into ampoules as crystals and sealed without further desiccation. Since this method of ampouling had not caused detectable chemical modification, a collaborative study of the crystalline porcine, bovine, and human insulins, together with a preparation of human insulin made by recombinant DNA procedures, had been arranged.
20. Parathyroid Hormone 1-34 Peptides, Bovine and Human

The Committee noted that, in accordance with the request made in its thirty-third report (WHO Technical Report Series, No. 687, 1983, p. 29), the National Institute for Biological Standards and Control, London, had obtained and distributed into ampoules synthetic preparations of the 1-34 peptides of the sequences of bovine and human parathyroid hormones (WHO/BS/83.1404). The Committee noted also that both substances had been included for evaluation and calibration in the international collaborative study of the proposed replacement of the International Reference Preparation of Parathyroid Hormone, Bovine, for Bioassay.

21. Proinsulins and Human Insulin C-Peptide

The Committee noted that, in accordance with the request made in its thirty-third report (WHO Technical Report Series, No. 687, 1983, p. 27), the National Institute for Biological Standards and Control, London, had obtained a preparation of human proinsulin, and that a quantity of human insulin C-peptide was available (WHO/BS/83.1400). Each preparation had been made by a recombinant DNA procedure because it had not been possible to obtain sufficient materials from natural sources. The Committee noted also that preparations of bovine and porcine proinsulins had been offered as proposed reference materials.

The Committee requested the National Institute for Biological Standards and Control, London, to obtain the materials, to distribute them into ampoules, and to arrange international collaborative studies.

22. Prolactin, Human, for Immunoassay

The Committee noted that the collaborative study of a proposed replacement preparation of human prolactin, mentioned in its thirty-second report (WHO Technical Report Series, No. 673, 1982, p. 30), had shown, in all the assay systems included, that the material was not suitable to serve as an international reference preparation of prolactin, human, for immunoassay (WHO/BS/83.1401).

The Committee noted that because stocks of the International Reference Preparation of Prolactin, Human, for Immunoassay, were
almost exhausted, the candidate preparation, coded 81/541, was being made available on an interim basis. The Committee requested the National Institute for Biological Standards and Control, London, to obtain suitable replacement material and to arrange a collaborative assay.

23. Thyroid Stimulating Hormone, Human, for Immunoassay

The Committee noted that the National Institute for Biological Standards and Control, London, had completed an international collaborative study which had included assay by both in vivo and in vitro methods of several preparations of thyroid stimulating hormone (WHO/BS/83.1402). The Committee was informed that, in accordance with the request made in its thirty-third report (WHO Technical Report Series No. 687, 1983, p. 28), the National Institute for Biological Standards and Control had established the preparation coded 80/558 as the second International Reference Preparation of Thyroid Stimulating Hormone, Human, for Immunoassay, and, on the basis of the results of the collaborative assay and with the agreement of the participants, had assigned an activity of 0.037 International Unit of Thyroid Stimulating Hormone, Human, for Immunoassay, to the contents of each ampoule.

MISCELLANEOUS SUBSTANCES

24. Endotoxin

The Committee was informed that the National Center for Drugs and Biologics, Bethesda, had offered a quantity of the material from which the USA national standard for endotoxin had been prepared. The Committee was informed also that the material would be freeze-dried in ampoules, and that an international collaborative study would be organized by the National Institute for Biological Standards and Control, London, in which it would be compared with the ampoules of freeze-dried material prepared from the International Reference Preparation of Pyrogen (WHO/BS/83.1426) and the USA national standard for endotoxin, with a view to establishing an international standard for endotoxin with an assigned unitage.
25. Human Autoantibody Sera for Pattern Recognition in Immunofluorescence Tests

The Committee noted that there was a clinical need for reference sera for pattern recognition of human autoantibodies in immunofluorescence tests, and that the provision of such sera, showing defined patterns of staining, would help to meet that need (WHO/BS/83.1390). Two preparations, anti-nuclear ribonucleoprotein serum and anti-smooth muscle serum, had been subjected to indirect immunofluorescence tests on a variety of tissues. The materials had been shown to be specific and stable.

The Committee therefore established the preparations, provided and studied by the Standardization Committee of the International Union of Immunological Societies, as the International Reference Reagent of Human Anti-Nuclear Ribonucleoprotein Serum and the International Reference Reagent of Human Anti-Smooth Muscle Serum.

26. Rubella Virus (Strain M-33) and Rubella Antiserum, Rabbit

The Committee was informed that the wild rubella virus (Strain M-33) was particularly suitable for use in a serum neutralization test, as well as in a haemagglutination inhibition test. Accordingly, the offer by the National Institute of Health, Tokyo, to supply ampoules of a freeze-dried suspension of the virus as a reference reagent had been accepted. The preparation had been shown to be stable and had given consistent results in several tests in a limited international collaborative study.

The National Institute of Health had also prepared an antibody to virus M-33 which had been shown to be useful in the haemagglutination inhibition test. This antiserum had also been distributed into ampoules, freeze-dried, and subjected to a limited international collaborative study.

The Committee agreed that these preparations would be useful reagents, and established the freeze-dried virus suspension as the International Reference Reagent of Rubella Virus (Strain M-33) and the antiserum as the International Reference Reagent of Rubella Antiserum, Rabbit.

The Committee requested the WHO Secretariat to arrange a collaborative study to assess the suitability of the International
Reference Reagent of Rubella Antiserum, Rabbit, in a serum neutralization test.

27. **Snake Venoms and Antivenoms**

The Committee noted the progress made in the characterization of snake venoms since its thirty-third meeting (WHO Technical Report Series, No. 687, 1983, p. 8). It noted also that quantities of six of the eight venoms selected for initial study had been received by the WHO Collaborating Centre for the Control of Venoms and Antivenoms, Liverpool School of Tropical Medicine, Liverpool, and had been characterized, together with many other venoms, by several biochemical and *in vivo* methods (WHO/BS/83.1403).

The Committee was informed that the six venoms were to be distributed into ampoules and used to prepare antivenoms which would be studied for their suitability to serve as international reference materials.

**REQUIREMENTS FOR BIOLOGICAL SUBSTANCES**

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

The Committee noted that immune precipitation tests in gels had been used for many years for establishing the identity of toxoids in vaccines containing diphtheria and tetanus toxoids (WHO/BS/83.1420). The Committee agreed that the tests should be included in the relevant international requirements.

The Committee adopted the proposed addendum to the *Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines* and agreed that it should be annexed to this report (Annex 1).

29. **Requirements for Rift Valley Fever Vaccine (Live, Attenuated) for Veterinary Use**

The Committee was informed that Rift Valley fever was a disease normally confined to cattle and sheep but that, in 1977, the virus had
spread to man in Egypt and had become a serious public health hazard. In order to decrease the source of infection markedly, a safe, effective, and inexpensive vaccine for use in cattle and sheep was needed. The Committee was informed also that studies had shown that the Smithburn strain of Rift Valley fever virus was suitable for the preparation of a vaccine that fulfilled those criteria, and that requirements had been formulated for its production and control (WHO/BS/83.1392). The requirements had been distributed for comment and revised in the light of those received (WHO/BS/83.1392 Rev. 1).

The Committee considered that the tests applied had been adequate and, in view of the need, adopted the Requirements for Rift Valley Fever Vaccine (Live, Attenuated) for Veterinary Use and agreed that they should be annexed to this report (Annex 2).

30. Requirements for Typhoid Vaccine (Live Attenuated, Ty 21a, Oral)

The Committee was informed that, although some presently available killed typhoid vaccines gave immunity, they were not wholly satisfactory, particularly because they gave rise to undesirable local and systemic reactions. Several studies with a Salmonella typhimurium mouse typhoid model system had clearly demonstrated that vaccination with living attenuated Salmonella produced a more effective immunity than did killed vaccine given parenterally.

The Committee noted that a live Ty 21a vaccine strain given by the oral route had been demonstrated to be safe in several studies, that a course of three doses of the vaccine had been shown to be protective, and that a group of consultants had formulated requirements for typhoid vaccine (live attenuated, Ty 21a, oral) (WHO/BS/82.1389). The Committee noted also that the requirements had been distributed for comment and that appropriate amendments had been incorporated in the final document (WHO/BS/82.1389 Rev. 1). The Committee considered that the tests applied had been relevant and noted that no reliable potency test was at present available other than the count of viable bacteria.

In view of the need for requirements, the Committee adopted the Requirements for Typhoid Vaccine (Live Attenuated, Ty 21a, Oral) and agreed that they should be annexed to this report (Annex 3).
31. Biological Products made by Recombinant DNA Techniques

The Committee noted that, following discussions at its thirty-first, thirty-second, and thirty-third meetings (WHO Technical Report Series, No. 658, 1981, p. 11; No. 673, 1982, p. 16; and No. 687, 1983, p. 15), a WHO consultation had been held on the quality control of biologicals produced by recombinant DNA techniques. The basic principles of the techniques involved had been discussed, the tests already being applied to biologicals produced by such techniques had been reviewed, and an explanatory article had been published. Although there was a need for further discussion in regard to the provision of appropriate reference materials, and the tests to be applied during production and to the final products, the Committee agreed that a useful start had been made. The Committee agreed also that veterinary products made by recombinant DNA techniques should be considered.

32. Intravenous Immunoglobulins

The Committee noted that the clinical value of immunoglobulins (human) prepared for intravenous use had been stressed at an informal meeting on the subject. Intravenous administration made these immunoglobulins more rapidly available than did intramuscular injection, caused less pain, and led to fewer adverse reactions (WHO/BS/83.1396). The Committee noted also that the use of intravenous immunoglobulins, with their improved bioavailability, was increasing. It requested the WHO Secretariat to formulate requirements as soon as it became possible to do so.

ACKNOWLEDGEMENTS

The Committee thanks the following WHO staff members for their special contributions to its deliberations: Mr D. A. Lowe, Technical Terminology Service; Dr P. Sizaret, Biologicals.

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Annex 1

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

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

Addendum 1983

(1) In the revised Requirements (WHO Technical Report Series, No. 638, 1979, pp. 37–115) it is suggested that the control test for identity on the final product for diphtheria toxoid and for tetanus toxoid, whether singly or as a combined (DT or DPT) vaccine, be made by flocculation of the toxoid with an appropriate antitoxin.

It is clear that, during the last few years, immune precipitation tests in gels have proved to be satisfactory for such tests and mention of them should be made in the Requirements.

For this reason, the following amendment is made to the Requirements as revised in 1978 (Diphtheria, p. 51; Tetanus, p. 92; and Combined Vaccines, p. 104).

Diphtheria and Tetanus: Section A.5.1 "Identity test"

Replace the first sentence in the suggested method by the following:

“Flocculation in solutions or immune precipitation in gels of the toxoid by specific antitoxin may serve as an identity test.”

(2) It has been demonstrated in several laboratories that, when the potency of the tetanus component of a combined triple (DPT) vaccine is measured in mice, the presence of the pertussis component potentiates the immune response to the tetanus toxoid. The interpretation of the potency test (Part A, Section A.9, pp. 103–104) carried out under these circumstances should, therefore, be expressed in a more precise manner.
Combined vaccines: Section A.9 "Potency test"

Replace the fourth paragraph by the following:

"The potency of the tetanus component shall not be less than 40 IU per single human dose if the test is performed in guinea-pigs. If mice are used, and if the reference vaccine used for comparison does not contain a pertussis component, the potency of the tetanus component shall not be less than 60 IU per single human dose. The 95% confidence interval of the tests shall be smaller than 50–200%.”

(3) Tetanus: Section A.3.3.4 (p. 87) "Detoxification and purification of toxin"

Small print, first paragraph

Replace the reference (11, Appendices T.6 and T.7) with (11, Appendix T.11).
Annex 2

REQUIREMENTS FOR RIFT VALLEY FEVER VACCINE (LIVE, ATTENUATED) FOR VETERINARY USE

(Requirements for Biological Substances No. 35)

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1 Prepared by: Dr F. G. Davies, Veterinary Research Department, Kabeta, Kenya; Dr F. T. Perkins, Chief, Biologicals, WHO, Geneva, Switzerland.
GENERAL CONSIDERATIONS

Requirements for Rift Valley Fever Vaccine (Inactivated) for Human Use were formulated in 1981, (WHO Technical Report Series No. 673, 1982) because the disease, normally confined to cattle, had spread to man and had become a public health hazard in Egypt in 1977. The logical step to take is to attempt to eliminate the source of the disease but for this it is necessary to have vaccine available in large quantities. Live vaccine, made from an attenuated strain that has been shown to be safe and effective in field studies, offers the best hope of reducing the incidence of the disease in cattle.

The cost-effectiveness of any prophylactic vaccination programme for domestic animals in the developing countries of Africa is of critical importance. Whereas 1 ml of inactivated tissue culture vaccine may be needed for a single inoculation, the same volume of an attenuated strain may contain up to 10,000 doses. This is an important consideration.

The Smithburn strain of Rift Valley fever is the most widely used modified live virus vaccine (MLVV). In the cattle and sheep breeds indigenous to Africa, this strain has been shown to be both safe and immunogenic. Some strains or breeds of sheep exotic to the continent, however, may show foetal abnormalities if vaccinated during pregnancy. Planned vaccination programmes, which ensure that pregnant animals are not vaccinated, avoid this problem completely. During epizootics, however, the losses caused by the disease are far greater than those associated with vaccination of pregnant sheep.

The Smithburn strain grows in suckling mouse brain and in various tissue cultures to high titres of virus. The tissue culture systems offer a more controllable growth system for vaccine production and are to be preferred. No differences have been detected between the immunogenicity of mouse brain- and tissue culture-prepared vaccines.

The Smithburn strain, therefore, has considerable value for prophylaxis against Rift Valley fever in cattle and sheep in Africa, where the virus is widespread. Until such time as a better modified strain becomes available for the preparation of live virus vaccine, its minor disadvantages must be accepted and avoided by careful use.

As with the requirements for vaccines that have been formulated or revised during the last five years, a protocol for reporting the results of all tests has been included (see Appendix). Completion of such a protocol, which is based on the present requirements, enables
a control authority to check whether all the tests have been completed.

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

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning Rift Valley fever vaccine (live attenuated), it is recommended that a clause be included permitting modifications of manufacturing requirements on condition that it can 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. The World Health Organization should then be informed of the action taken.

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

PART A.

MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be “Vaccinum febris Rift Valley vivum (ad usum veterinarium)”. The proper name shall be the equivalent of the international name in the language of the country of origin.

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

1.2 Descriptive definition

“Vaccinum febris Rift Valley vivum (ad usum veterinarium)” shall consist of freeze-dried Rift Valley fever virus grown in cell
cultures or in mouse brains. The preparation shall satisfy all the requirements formulated below.

1.3 Terminology

*Virus seed lot*: A quantity of virus processed together and of uniform composition. In each manufacturing establishment a *primary virus seed lot* is that from which further *virus seed lots* are prepared. Vaccine may be up to 5 passages from the primary seed lot.

*Cell seed*: A quantity of cells derived from a single tissue and of uniform composition, stored frozen at \(-70^\circ C\) or below in aliquots, one or more of which would be used for the production of a manufacturer's working cell bank (MWCB).

*Manufacturer's working cell bank (MWCB)*: All of the cellular material derived by serial subculture from one or more ampoules of cell seed. After serial subculture the cells are combined into a pool and preserved cryogenically to form the MWCB. One or more ampoules from a pool would be used for the production of a single harvest.

*Single harvest*: A virus suspension harvested from cell cultures or the brains of suckling mice that has been derived from a single batch of cells or group of mice and processed together.

*Single pool*: A virus suspension processed at the same time.

*Final bulk*: The finished biological preparation 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. A filling lot must, therefore, have been filled in one working session from a single final bulk.

*Tissue culture infective dose 50% (TCID_{50})*: The quantity of a virus suspension that will infect 50% of inoculated cell cultures.

*Suckling mouse intracerebral lethal dose 50% (MLD_{50})*: The quantity of virus that will kill 50% of inoculated suckling mice.

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)_ (WHO
Technical Report Series, No. 323, 1966, p. 11) shall apply to establishments manufacturing Rift Valley fever (RVF) vaccine (live, attenuated), with the addition of the following directives:

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

The production of RVF vaccine shall be conducted by staff who have not handled other infectious microorganisms or animals on the same working day. The staff shall consist of persons who shall 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 RVF virus.

No culture of any microorganisms or cells other than the cultures approved by the national control authority for the production of RVF vaccine shall be introduced or handled in the production area at any time during manufacture of the vaccine.

Persons not directly concerned with the production process shall not be permitted to enter the production area.

3. PRODUCTION CONTROL

3.1 Control of source materials

3.1.1 Virus strains and seed lot system

The strain of RVF used in the production of vaccine shall be approved by the national control authority. It shall be identified by historical records, by infectivity tests, and by immunological methods. Production of vaccine shall be based on the seed lot system; the seed virus used for vaccine production shall not have passed more than 4 subcultures either in suckling mouse brain or in approved cell cultures counted from a primary seed lot used for the production of the vaccine on which the original laboratory and field tests were done.

The primary seed lot should also be shown to be free from wild Rift Valley fever virus.

Samples of the strain used shall be deposited in the national control laboratory.

The Smithburn neurotropic strain at the 104th mouse brain passage has been shown to be safe and immunogenic for cattle and non-pregnant sheep.
3.1.2 *Cell substrate*

The virus growth used for vaccine may be made in cell cultures or in suckling mouse brain.

3.1.2.1 *Cell cultures*

The cell culture used for the preparation of the manufacturer's working cell bank shall be approved by the national control laboratory.

Suitable cells for this purpose are BHK21c13, VERO, or CER cells.

The cell bank from which the cells are grown shall be shown to be free from infectious agents, particularly bovine virus diarrhoea, bluetongue, and infectious bovine rhinotracheitis, as well as wild Rift Valley fever.

3.1.2.2 *Suckling mouse brain*

The suckling mice used for the growth of RVF shall be obtained from a healthy colony of mice. They shall be kept in quarantine and monitored continuously. They shall be free from signs of disease and shown to be free from murine viruses. The mouse colony shall also be shown to be free from *Salmonella*.

3.1.3 *Cell culture medium*

Serum used for the propagation of cells for vaccine production shall be tested to demonstrate freedom from bacteria, fungi, and mycoplasma, according to the requirements given in Part A, sections 5.2 and 5.3 of the revised *Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances)* (WHO Technical Report Series, No. 530, 1973, pp. 49–52) as well as freedom from pathogens of the species of the origin of the serum by methods approved by the national control authority.

In some countries sera are examined for freedom from phage.

Suitable antibiotics in minimum concentrations required for sterility may be used. Nontoxic pH indicators may be added, e.g., phenol red in a concentration of 20 mg/l.
3.1.4 Tests on virus seed lots

Each virus seed lot shall be subjected to all tests applicable to a single pool and final lot (see Part A, sections 3.2, 3.4.1, 3.4.2, 5.3, 5.4, 5.5, and 5.6). The national control authority shall approve the virus seed lots.

The virus for vaccine production shall be tested for murine viruses.

The virus seed lot shall have a RVF virus content greater than 7.5 log_{10} TCID_{50}/ml or MLD_{50}/ml.

3.2 Control cells for vaccine prepared in cell cultures

A cell sample equivalent to at least 1% of the cell suspension, at the concentration employed for vaccine production cultures, shall be used to prepare control cell cultures.

In some countries in which the technology of large-scale production has been developed the national control authority should determine the size of the sample of cells to be examined, the time at which the control cells should be taken from the production culture, and the monitoring of the control cultures.

These control cell cultures shall be incubated for at least two weeks 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 this time, the control cell cultures shall be examined for any evidence of degeneration caused by extraneous agents.

If there is any cytopathic effect attributable to any extraneous agent in the supernatant fluid or the control cultures, the virus grown on the same batch of cells shall not be used for vaccine production.

3.2.1 Tests for haemadsorbing viruses

At the end of the observation period or at the time the virus is harvested from the production cultures, whichever is the later, 25% of the control cells shall be tested for the presence of haemadsorbing viruses, using guinea-pig red cells. If the guinea-pig red cells have been stored, the duration of storage shall not have exceeded 7 days, and the temperature of storage shall have been in the range of 2–8 °C. The cells shall be tested also for Rift Valley fever virus.
Guinea-pig red cells are usually used for this test. In some countries the national control authority requires that additional tests for haemadsorbing viruses should be made using other types of red cell, including those from man (blood group 0), monkeys, and chickens (or other avian species).

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

In countries in which there is foot and mouth disease it is suggested that the cells be tested for foot and mouth virus.

IF ANY HAEMADSORBING VIRUS OR RIETF VALLEY FEVER VIRUS IS FOUND, THE VIRUS HARVESTED FROM THAT BATCH OF CELLS SHALL NOT BE USED FOR THE PREPARATION OF VACCINE.

3.3 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) (WHO Technical Report Series, No. 323, 1966, p. 15) shall apply to the manufacture of RVF vaccine (live attenuated). If animal serum is used for the growth of cell cultures, the serum concentration in the final vaccine shall be not more than 1 μg/l. The serum concentration shall be reduced to this level by rinsing the cell cultures with serum-free medium and/or purification of the virus harvests.

In some countries, control tests are carried out to detect the residual animal serum content in the final vaccine (see Part A, section 5.5).

3.3.1 Inoculation of suckling mice

When suckling mice are used for the production of vaccine, each healthy suckling mouse between 2 and 6 days old shall be inoculated by the intracerebral route with an inoculum of 0.025 ml containing 1 : 100 dilution of the seed lot in 5% peptone at pH 7.4, containing 200 μg of penicillin and 200 μg of streptomycin sulfate per millitre.

3.3.2 Inoculation of cell cultures

The cell cultures shall be inoculated with a 1 : 100 000 dilution of the seed lot made in maintenance medium without serum. The virus
shall be allowed to become adsorbed into the cells at 37 °C for one hour before adding maintenance medium to the cultures.

The initial inoculum should not exceed 0.1 ml per square cm of cells.

3.3.3 Harvesting of mouse brains

Suckling mice that are sick or dying on the second day after inoculation shall be held at −20 °C and all further inoculated mice shall be placed at a similar temperature 3 days post-inoculation.

The mouse brains shall be harvested aseptically and emulsified in 5% peptone containing a buffer at pH 7.4 to make a 10% suspension.

Such suspensions may be frozen once only as single harvests and later pooled to form the final bulk. The storage period between harvesting and pooling should be as short as possible.

3.3.4 Harvesting of cell cultures

The supernatant fluids of cell cultures shall be harvested when the cells show a cytopathic effect of 50–70%. The virus harvest shall be centrifuged at 600 g to remove cell debris and the virus suspension diluted in 5% peptone containing a buffer at pH 7.4 to give the appropriate virus titre.

Use of other suitable stabilizing agents may be permitted.

The pooled virus suspensions from a single batch of cells form a single harvest which may be frozen at −20 °C for a short time and blended with other single harvests to form the final bulk.

3.4 Control of single harvests

At the time of freezing the single harvests, samples shall be taken and subjected to the following tests.

3.4.1 Sterility test

Each single harvest shall be tested for sterility according to the requirements given in Part A, sections 5.1, 5.2, and 5.3, of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (WHO Technical Report Series, No. 530, 1973, pp. 48–52).
3.4.2 Identity test

The virus in the single harvest shall be tested for identity by the direct fluorescent antibody test or neutralization of the virus with specific antiserum.

3.4.3 Virus titre

Each single harvest shall be shown to have a satisfactory virus content by the inoculation by the intracerebral route of 2–4-day-old mice.

3.5 Pooling of single harvests

As soon as each single harvest to be pooled into a particular final bulk has been shown to be satisfactory and the control cultures of vaccines made in cell cultures have also been shown to be free from extraneous agents (see Part A, section 3.2), the single harvests shall be thawed and blended aseptically to form the final bulk.

The final bulk shall be filled without further storage into ampoules, and freeze-dried to form the filling lot (final lot).

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) (WHO Technical Report Series, No. 323, 1966, p. 16), shall apply, with the addition of the following directive.

The containers shall be of colourless and fully transparent glass so as to permit the detection of any changes in the appearance of the vaccine.

Single- and multiple-dose containers may be used.

5. CONTROL TESTS ON FINAL PRODUCT

5.1 Identity test

An identity test shall be done on at least 1 labelled container from each filling lot by an appropriate method (see Part A, section 3.4.2).
5.2 Sterility tests

Each filling lot shall be tested for sterility according to the requirements given in Part A, sections 5.1 and 5.2 of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (WHO Technical Report Series, No. 530, 1973, pp. 48–52).

5.3 Virus titre

Each filling lot shall be shown to have a satisfactory virus content by the inoculation by the intracerebral route of 2–4-day-old mice. The national control authority shall determine the immunizing dose.

5.4 Safety tests

After reconstitution of the vaccine, in phosphate buffer at pH 7.4, to the concentration in which it shall be used, ampoules from each final lot shall be subjected to 3 safety tests as follows:

5.4.1 Inoculation of hamsters

At least 2 hamsters shall be inoculated by the intraperitoneal route with 0.5 ml of the vaccine. The hamsters shall be observed for 21 days and shall remain well.

5.4.2 Inoculation of sheep

Each of 4 sheep shall be shown by a neutralizing antibody test to be free from RVF, bluetongue, Nairobi sheep disease, and orf, as well as sheep and goat pox. Two sheep shall be inoculated by the intravenous route and 2 by the subcutaneous route; each inoculum shall be the total content of a single ampoule.

It is suggested that in countries with foot and mouth disease the sheep should be shown to be free from foot and mouth antibody.
The sheep shall be examined for a period of 21 days for febrile reaction or signs of clinical disease. At the end of the observation period the animals shall be bled, and the serum tested for the presence of antibodies to RVF virus and for the absence of antibodies to any of the infectious agents mentioned above.
There shall be shown to be a response to RVF virus and none to other infectious agents.

5.4.3 Inoculation of cattle
Each of 2 cattle of 1–2 years of age, which have been shown to be free from RVF antibody, shall be inoculated by the subcutaneous route with the vaccine dose. The cattle shall be examined for a period of 21 days for febrile reaction or other signs of clinical disease.
All animals shall survive the tests and in the event of any reaction shown to be caused by the vaccine the final lot shall be rejected.

5.5 Potency test
The test for virus titre in mice (see Part A, section 5.3) is a measure of potency.

The vaccine dose for sheep is 10 000 MLD_{10} and that for cattle 20 000 MLD_{30}. The equivalent dose should be determined when the titre is made in cell cultures (TCID_{50}).

5.5.1 Test in non-pregnant sheep
Each of 8 non-pregnant sheep shall be shown to be free from RVF antibody; 4 sheep shall be inoculated with the vaccine dose and 4 with a dilution of 1 : 10. The neutralizing antibody titres measured in the serum 28 days later shall be at least 1 : 4 and the animals at that time shall resist a challenge of 4.0 log_{10} MLD_{50} of pantropic RVF virus.

5.5.2 Test in pregnant sheep
The test as outlined in Part A, section 5.5.1 shall be repeated in 4 pregnant sheep. In addition to stimulating an antibody response, the pregnant sheep shall not abort when challenged with the pantropic RVF virus.

In some countries, it is required that the challenged sheep shall have normal healthy lambs.

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5.6 Stability test

The vaccine in the freeze-dried form shall be incubated at 37°C for 7 days. After incubation it shall be reconstituted in 1% peptone phosphate buffer solution at pH 7.4 and the virus titre compared with that of an ampoule of vaccine that has not been incubated.

The vaccine shall not have lost more than 1.0 log_{10} MLD of virus on incubation and at the end of incubation shall retain the minimal immunizing dose for the animal in which it is intended for use.

On long-term storage the vaccine in the freeze-dried form should show little or no loss of virus titre for at least 24 months at −20°C.

The reconstituting fluid shall be distributed with the vaccine.

It has been shown that 1% peptone in phosphate buffer at pH 7.4 is the most suitable.

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) (WHO Technical Report Series, No. 323, 1966, p. 17) shall apply.

7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (WHO Technical Report Series, No. 323, 1966, p. 18) shall apply.

8. LABELLING

The requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (WHO Technical Report Series, No. 323, 1966, p. 18) shall apply, with the addition of the following directive.

The leaflet accompanying the package shall include the following information:
(i) the nature of the cell cultures used; or a statement that suckling mice have been used;
(ii) the virus strain used for the production of the vaccine;
(iii) a warning that the vaccine should be protected from light.

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) (WHO Technical Report Series, No. 323, 1966, p. 18) shall apply.

The vaccine shall be distributed at such a temperature that there is no loss of virus titre during distribution.

10. STORAGE AND EXPIRY DATE

10.1 Storage conditions

RVF vaccine (live attenuated) shall be stored at all times at −20°C or below.

10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall be not more than 24 months after the date of the last satisfactory potency test, the date of a potency test being that date on which the test animals were inoculated with the vaccine.

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

The national control authority shall give directions to manufacturers concerning the strain of RVF virus to use for production of vaccine.

The national control authority shall provide or approve the strain for challenge and the reference vaccine for use in the potency test (Part A, section 5.5).

2. RELEASE AND CERTIFICATION

A vaccine lot 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 lot of vaccine in question meets all national requirements as well as Part A of the present requirements. The certificate shall further state the date of the last satisfactory potency test, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of RVF vaccine (live, attenuated), for veterinary use between countries.

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Appendix

SUMMARY PROTOCOL FOR RIFT VALLEY FEVER VACCINE (LIVE, ATTENUATED) FOR VETERINARY USE
PRODUCTION AND TESTING

(Based on Requirements for Biological Substances No. 35, Requirements for Rift Valley Fever Vaccine (Live, Attenuated) for Veterinary Use, WHO Technical Report Series, No. 700, 1984, Annex 2)

Identification of Final Lot

Name and address of manufacturer

Lot number of final product

Date of manufacture of final lot (namely, date of last potency test)

Expiry date

Total volume of final lot

Cell Cultures

Type of cell cultures used:

Origin and short history of cell seed

Authority that approved cell seed

Amount of cell culture inoculated

Amount of control cell culture investigated

Tests performed on control cultures

Result

Serum for Cell Cultures

Origin of serum used

Tests performed on serum

Result

44
Mouse Colony

Strain of mouse colony
Number of mice per litter

Seed Lot of Virus

Strain used
Origin and source of strain
Number of subcultures between primary seed lot and production

Information on Manufacture

Single harvests
1. Date of inoculation of cells with virus
   Date of virus harvest
   Special observations on single harvests
   Date of pooling

2. Date of inoculation of suckling mice
   Number of mice inoculated
   Date of virus harvest
   Date of pooling

Tests on single harvest

Sterility test
   Method (media)
   Result

Identity test
   Method
   Result

Tests on final product

Identity test
   Method
   Result

45
**Sterility test**
- Method (media)
- Result

**Virus titre**
- Method
- Result

**Safety tests**
1. **In hamsters**
   - No. inoculated
   - Result

2. **In sheep**
   - Freedom from infections
   - Period of examination
   - Pyrexia
   - Infection
   - Absence of antibodies to other viruses
   - RVF neutralizing titres

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<th>Sheep i.v.</th>
<th>Sheep s.c.</th>
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3. **In cattle**
   - Pyrexia
   - Infection

**Potency test**
- No. of MLD per ampoule
- No. of doses per ampoule

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1. **Test in non-pregnant sheep**
   - Antibody response in sheep inoculated with 1:1 vaccine with 1:10 vaccine
   - Result of challenge
   - Dose of challenge (MLD₅₀)
   - Result

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2. *Test in pregnant sheep*

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<th>No. 3</th>
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<tr>
<td>Antibody response of sheep inoculated with 1:1 vaccine</td>
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<tr>
<td>with 1:10 vaccine</td>
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<tr>
<td>Result of challenge</td>
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<tr>
<td>Dose of challenge (MLD&lt;sub&gt;50&lt;/sub&gt;)</td>
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<tr>
<td>Result</td>
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</tbody>
</table>

*Stability test*

Loss of virus titre (MLD<sub>50</sub>) on incubation

Signature of head of laboratory

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

I certify that lot No. ... of Rift Valley Fever Vaccine (Live, Attenuated) for Veterinary Use satisfies the WHO Requirements for Rift Valley Fever Vaccine (Live, Attenuated) for Veterinary Use.

Date

Signature

Name typed

The protocol must be accompanied by a sample of the vaccine and a copy of the leaflet.
Annex 3

REQUIREMENTS FOR TYPHOID VACCINE
(LIVE ATTENUATED, Ty 21a, ORAL)

(Requirements for Biological Substances No. 34)

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INTRODUCTION

Many health authorities require a vaccine against typhoid fever and international requirements for such a prophylactic are essential.

The formulation of requirements was first considered at the twelfth WHO Expert Committee on Biological Standardization, over 20 years ago. At that time, however, there were few satisfactory laboratory tests available for the control of such vaccines. It was several years before any requirements could be formulated. The many field trials, showing efficacy of some of the vaccines, gave important leads towards meaningful tests to be embodied in requirements and these were formulated and accepted in 1966.

Such requirements concerned only the vaccine made from Salmonella typhi Ty 2, killed by chemicals and given by the parenteral route. Although it was recognized that there was no satisfactory laboratory test for potency, a test in which immunized mice were challenged was included as the best available.

With the recent advancement made in the isolation of an avirulent mutant that can be given orally as a live vaccine, and its proven success in a field trial, the Sub-Committee of the Scientific Working Group on Bacterial Enteric Infections expressed the need for requirements specifically concerned with such a strain. Since there are no other mutants under consideration, and since the tests characterizing the strain are unique, the requirements have been formulated exclusively for the strain known as Salmonella typhi Ty 21a.

Having regard to these considerations, the following international requirements for typhoid vaccine (live attenuated, Ty 21a, oral) have been formulated and fitted into the framework adopted in the Requirements for Biological Substances Nos. 1 to 32 already published by WHO (1). In drafting these requirements, account has been taken of the opinions of consultants as well as information from published and unpublished reports. In addition, opinions and data have been received from a number of experts, whose assistance is gratefully acknowledged below.

GENERAL CONSIDERATIONS

Presently available typhoid vaccines are not wholly satisfactory. Although some killed vaccines given by the parenteral route have been shown, in large controlled field trials sponsored by WHO, to
confer good and long-lasting protection, they tend to cause undesired local and systemic reactions. Furthermore, as has been demonstrated in volunteer challenge studies, the protection is not absolute but can be overwhelmed by large challenge doses. Oral killed vaccines, on the other hand, are well tolerated but their efficacy could never be demonstrated in volunteer challenge studies or controlled field trials. They cannot therefore be recommended.

Several studies with a Salmonella typhimurium-mouse typhoid model have clearly demonstrated that vaccination with live attenuated Salmonella produces a more effective immunity than does parenteral killed vaccine. The superiority of attenuated live vaccines is particularly well demonstrated when vaccine and challenge are administered by the natural oral route. Based on the results obtained with such an animal model, an attenuated Salmonella typhi has been developed and prepared as a candidate for an oral live typhoid vaccine strain. The main characteristic of this Salmonella typhi mutant (strain Ty 21a) is the lack of the enzyme uridine-disphosphate-galactose-4-epimerase; therefore, biosynthesis of the cell wall lipopolysaccharides, which are responsible for both immunogenicity and virulence, occurs in this mutant only under conditions that induce bacteriolysis and thereby inactivation of the bacteria.

The safety of the Ty 21a vaccine strain has been proved by volunteer challenge studies in the United States of America, by a large field trial in Egypt, and in a pilot study in Chile. In the study in the United States of America, 5–8 doses of vaccine containing 3–10 × 10^10 viable bacteria per dose were given to 155 adult males without significant side-effects. Three doses of vaccine containing 1–8 × 10^9 viable bacteria were given to 16,000 schoolchildren in Egypt and 3 doses containing 1–5 × 10^9 viable bacteria were given to 338 schoolchildren in Chile in the pilot study and to about 60,000 children in a larger trial in Chile, again with no ill effects.

As far as excretion is concerned, the vaccine strain in stools of volunteers given the high doses (3–10 × 10^10) was low and was never excreted for more than three days. The majority of isolates occurred on the first day after vaccination. None of the 358 stool isolates examined showed any sign of reversion to virulence. The vaccine strain could not be isolated from the stools of the Egyptian and Chilean schoolchildren who received the lower vaccine dosage (1–8 × 10^9).

Furthermore, the protective efficacy was demonstrated in both the
volunteer challenge studies in the United States of America and the field trial in Egypt. In the volunteer studies, the vaccine showed an efficacy of 87% against a challenge dose that provoked typhoid fever in 57% of the control group. In earlier studies using killed vaccine given parenterally, no protection was demonstrated against such a high challenge dose. In addition to protecting against the disease, Ty 21a vaccine also shortened the excretion period of the *Salmonella typhi* challenge strain in the stools, compared with that of the non-vaccinated subjects. In the Egyptian field trial, in which the population was carefully monitored for three years, the efficacy was 96%. These results indicate that, in the dose schedules and formulation used, the Ty 21a vaccine is stable and safe, and is highly protective for a period of at least three years.

The only difficulty encountered is that, in order to protect the vaccine bacteria from inactivation by gastric acid, the gastric juice has to be neutralized by sodium bicarbonate before the vaccine is given. The Ty 21a vaccine at present available in several countries of Europe, South America, and Asia is administered in three doses given every second day; each dose consists of one capsule containing the vaccine strain and two capsules each containing 0.4 g of sodium bicarbonate. In May 1982 a field trial was launched in Chile in order to investigate if a more practical form of vaccine, consisting of only one or two enteric-coated capsules, each containing $1-5 \times 10^9$ Ty 21a bacteria, would be as effective as the formulation used in Egypt which was not in the form of enteric-coated capsules.

As there is no reliable potency test available to predict the efficacy of typhoid vaccines in man, more research is necessary and clinical trials must continue. Furthermore, since there is no animal model to give guidance on the safety of a strain, emphasis must be placed on the biochemical and immunological characterization of those strains that have been shown by clinical trials to be safe and effective in man.

Since no other mutants are being studied, the present requirements relate to a vaccine intended for oral administration and made only from the mutant Ty 21a.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements so that, if a health administration so desires, these parts as they appear may be included in definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance.
Should individual countries wish to adopt these requirements as the basis of their national regulations concerning live attenuated typhoid vaccine given orally, it is recommended that a clause be included permitting modifications of manufacturing requirements on 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 vaccine is manufactured.

PART A.
MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be Vaccum febris typhoidi (Ty 21a) perorale vivum. The proper name shall be equivalent to the international name in the language of the country of origin.

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

1.2 Descriptive definition

Live oral typhoid vaccine Ty 21a is a freeze-dried preparation contained in gelatin capsules prepared from the Salmonella typhi strain Ty 21a. Each capsule shall contain 1–5 × 10⁹ viable Ty 21a organisms (see Part A, section 3.3.1).

Enteric-coated capsules are being tested in field trials and these requirements apply also to such capsules.
1.3 International reference preparations and international units

There is a need for a freeze-dried international reference preparation of typhoid vaccine Ty 21a for comparison of the characteristics of the strain. The parent seed lot could serve this purpose.

1.4 Terminology

Parent seed lot: A quantity of living Salmonella typhi Ty 21a organisms derived from a single colony, processed together and of uniform composition. A parent seed lot shall be maintained in the freeze-dried form.

Working seed lot: A quantity of living Salmonella typhi Ty 21a organisms produced from the parent seed lot which is maintained in aliquots in the freeze-dried form.

Single harvest: One or more sediments obtained by centrifugation of cultures within 48 hours and harvested at the same time from an ampoule of the working seed lot.

Final bulk: A pool of single harvests freeze-dried in the same freeze-drying run and thereafter homogenized to form a uniform powder. The final bulk is contained in a single vessel.

Final lot: A collection of filled capsules that are homogeneous with respect to the risk of contamination during filling of one or more final bulks. A final lot must, therefore, have been filled in one working session.

2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control laboratories) (2) shall apply, with the addition of the following:

Production areas shall be decontaminated before they are used for the manufacture of typhoid Ty 21a vaccine.

Formaldehyde and glutaraldehyde have been found useful for this purpose.

The production of Ty 21a vaccine shall be conducted by a staff of healthy persons, who shall be examined medically at regular intervals. Steps shall be taken to ensure that all such persons in the
production area have been immunized against typhoid and do not excrete *S. typhi*.

The medical authority of the production area shall determine the frequency of the medical examinations.

Visitors and persons not directly concerned with the production process shall not be permitted to enter the production areas.

3. PRODUCTION CONTROL

3.1 Control of source materials

3.1.1 Bacterial strain

The attenuated *S. typhi* mutant strain Ty 21a has been shown to be safe and effective in man and has the characteristics described in Part A, section 3.1.4.

3.1.2 Production of parent seed lot

A single colony of organisms shall be grown using a suitable galactose-free medium and incubated at a temperature to produce luxuriant growth. As the culture reaches the stationary phase of growth the organisms shall be harvested, centrifuged, suspended in a stabilizer, dispensed into ampoules, and freeze-dried. Each ampoule shall contain at least $10^9$ viable organisms and satisfy the tests in Part A, section 3.1.4.

It has been found that brain heart infusion broth is suitable for this purpose.

The parent seed shall be tested according to the requirements in Part A, section 3.1.4. It shall be stored at $5^\circ$C ± 3°C.

3.1.3 Production of working seed lot

A working seed shall be produced from the growth of organisms obtained from an ampoule of the parent seed. The working seed shall be treated in a similar manner to the parent seed and freeze-dried. Each ampoule shall contain at least $10^9$ viable organisms.

The working seed shall be tested according to the requirements in Part A, section 3.1.4. It shall be stored at $5^\circ$C ± 3°C.
3.1.4 Tests on parent and working seed lots

The parent and working seeds shall satisfy the following tests:

3.1.4.1 Identity and purity

(a) The growth from the seeds shall confirm that the Ty 21a organisms are Gram-negative rods and that they are motile.

(b) Organisms grown on media for three passages with or without galactose agglutinate with H : d antiserum but not with Vi antiserum, whereas only those organisms grown on medium containing galactose (1 g/litre) will agglutinate with 0 : 9 antiserum.

(c) Samples, when plated on galactose containing indicator medium (for example Endo agar to which galactose has been added) and incubated at 37°C for 7 days, develop a coloured confluent growth which, due to lysis, gradually becomes transparent. Galactose-fermenting colonies shall not appear at any time during the 7 days' incubation.

The observation for 7 days is a sensitive test for reversion. Other indicator media such as bromthymol blue agar are also useful for this test.

(d) Samples plated on galactose containing indicator medium and incubated for 48 hours at 37°C shall have concave colonies with a pale border, due to active growth, and darker centres, due to death and lysis; such is a typical appearance of Ty 21a on galactose containing agar medium.

(e) Growth on Kliger iron agar slants shows no blackening, indicating that the organisms do not produce hydrogen sulfide.

The use of lead acetate paper is also suitable for the detection of H₂S.

3.1.4.2 Galactose-induced bacteriolysis

Shake-cultures growing in the exponential phase in heart infusion broth at 37°C shall begin to lyse within 1 hour of the addition of galactose at a final concentration of 100 g/litre. When a similar concentration of galactose is added at the time of inoculation the growth is limited before lysis takes place.

3.1.4.3 Mouse virulence

The organisms grown for 6 hours at 37°C in brain heart infusion broth shall be inoculated into 5 mice (18–20 g) by the intraperitoneal
route. A suspension containing at least $5 \times 10^7$ bacteria shall not kill any mice within a 7 day observation period.

The NIH-GI strain of mice are suitable for this test.

3.1.4.4 Determination of enzymes involved in galactose metabolism

Tests for the enzymes involved in galactose metabolism shall show that the Ty 21a organisms have lower enzyme activities than those of Ty 2.

When compared with the enzyme activity of Ty 2, taken as 100%, the activities of Ty 21a are epimerase 0%, galactokinase (EC 2.7.1.6) 5-20%, galactose-1-phosphate-uridylyltransferase (EC 2.7.7.10) 25-50%, and galactose-permease 40-50%.

The tests suitable for this test are mentioned in references (3-5).

3.1.4.5 Uptake and intracellular distribution of $^{14}$C galactose

The kinetics of the uptake and of the distribution of radioactive $^{14}$C galactose in the cells shall be shown to be typical for Ty 21a and shall differentiate it from other mutants free from epimerase.

After 7 hours’ growth at 30°C, at least 90% of the galactose in the medium (1 g/litre) shall be shown to have been taken up by the organisms by the measurement of $^{14}$C in the cells. It shall be shown also that approximately 75% of the galactose is in the cell wall.

It should be noted that the galactose uptake is not parallel to the growth curve and should be measured in 2 independent cultures at 1 hour intervals over a period of 7 hours.

3.1.4.6 Characterization of lipopolysaccharide (LPS)

Lipopolysaccharide extracted from the cell walls of the organisms grown in brain heart infusion broth containing 1 g/litre $^{14}$C galactose for 7 hours at 30°C, shall be hydrolysed in 1% acetic acid and the polysaccharides examined.

It shall be shown by gel chromatographic methods, using Sephadex G50, that the organisms contain LPS of both smooth type and rough type, in a similar ratio to that of the virulent strain S. typhi Ty 2.

The solvent system used for chromatography on Sephadex G50 of the polysaccharide portion of the LPS is pyridine acetate buffer at pH 5.4.
Some countries may permit the use of SDS-polyacrylamide gel electrophoresis for this test.

The rough LPS of *S. typhi* Ty 21a are of both Ra (complete core but no O-side-chain) and Rc (no O-side-chain and incomplete core without galactose) types, while Ty 2 rough LPS are of the Ra type only.

Paper chromatography of 14C-labelled LPS hydrolysed in 0.5 mol/l sulfuric acid shows that the 14C-label is present only on the galactose spot. (This is a sensitive test to show that the mutation in the epimerase gene is not leaky.)

Furthermore, it shall be shown that the distribution of the sugars keto-deoxy-octonate (KDO), galactose, glucose, and rhamnose of the LPS are similar in their ratios for both the Ty 21a and the Ty 2 strains.

### 3.2 Production precautions

The general production precautions, as formulated in the requirements of Part A, section 3, of the revised *Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)* (2) shall apply.

In addition, the production process from the growth of the working seed to the harvesting, centrifugation, and resuspension must be carried out in a closed system. The virulent Ty 2 strain shall be excluded from the production area.

#### 3.2.1 Culture medium for vaccine production

The culture medium used for vaccine production shall be free from ingredients that may cause toxic or allergic reactions in man when ingested.

#### 3.2.2 Temperature and time of incubation

The cultures shall be incubated at such a temperature and for such a time that they will be harvested in the early stationary phase.

The number of passages to provide the inoculum for the final fermenter from the working seed shall be kept to a minimum and in any event it shall not exceed 4 passages.

At every passage a sample of the bacterial suspension should be Gram stained and plated on blood agar to demonstrate the purity of the culture. In addition, plating on an indicator medium is used to confirm identity.
3.3 Control of single fermenters

Before centrifugation of the contents of the fermenters, samples shall be taken from each fermenter and tested to demonstrate that the bacteria have identity with the Ty 21a strain of the working seed, as shown by the tests of Part A, section 3.1.4.1 (c) and (d).

3.3.1 Viable count

The viable count of the contents of each fermenter shall be made on brain heart infusion agar and incubated at 37°C for 36 hours.

3.3.2 Pooling of contents of fermenters to form a single harvest

The contents of several fermenters may be centrifuged, resuspended in a stabilizer, and pooled to form a single harvest.

In some countries the contents of each fermenter are kept separate until after freeze-drying.

The stabilizer used shall be approved by the national control authority and the single harvests shall be maintained frozen until freeze-dried.

After freeze-drying, several single harvests may be blended to form the bulk powder, which is mixed thoroughly.

3.4 Control of final bulk

The final bulk is subjected to the following tests:

3.4.1 Test for identity

A sample of the powder shall be reconstituted and tested for identity by the test shown in Part A, section 3.1.4.1 (d).

3.4.2 Test for viable organisms

The viable count in a weighed quantity shall be measured by the test formulated in Part A, section 3.3.1.

The viable count shall be compared with that of the single harvests stored before freeze-drying and a minimum of 10% of the organisms shall have survived the freeze-drying process.
3.4.3 Test for residual moisture

The final bulk shall be tested for residual moisture which shall be shown to be not greater than a level approved by the national control authority.

Two methods have been shown to be useful. When the Karl Fischer method is used with Aquavit-Tacussel equipment the moisture content should be less than 3%.

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 to vaccine filled in the dried form.

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

4.1 Filling into capsules

The final bulk shall be homogenized with a calculated quantity of dry filling material such that at least $2.5 \times 10^9$ live organisms shall be filled into each gelatin capsule.

The range of $2.5 \times 10^9$ is to ensure that the capsules contain at least $1 \times 10^9$ as the human dose at the time the subject takes the capsule.

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 contents of 3 capsules shall be tested for identity by the test described in Part A, section 3.1.4.1 (d).
5.2 Viable count

The viable count of the contents of 5 capsules shall be determined by the test described in Part A, section 3.3.1; it shall be between 2 and \(5 \times 10^9\) viable organisms per capsule. This test of viable count shall be regarded as the test for potency.

A suitable method is to empty the contents of at least 5 capsules into a flat-bottom sterile Kolle-type flask with glass beads and then to suspend in 20 ml or 50 ml of physiological saline. The suspending is accomplished by swirling for approximately 15 minutes on a rotary shaker.

Serial dilutions are prepared in physiological saline and—according to the presumed live count of capsules—the appropriate dilutions are plated on at least 5 brain heart infusion agar plates per dilution, with each plate receiving 0.1 ml. After aerobic incubation at 35–37 °C for 30–36 hours or longer, the typical colonies are counted and the average live cell content of each capsule is calculated.

5.3 Test for freedom from gross contamination

The contents of at least 3 capsules shall be tested on selective media for freedom from pathogens such as *Salmonellae* (other than Ty 21a), *Shigella, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Vibrio parahaemolyticus*. It shall be shown that none is present.

In addition, the number of non-pathogenic organisms that may be present shall not exceed \(2 \times 10^5\) bacteria and 20 fungi per capsule.

5.4 Innocuity test

Each final lot shall be tested for abnormal toxicity by the inoculation of 0.01 human dose intraperitoneally into each of 5 mice and by giving a human dose orally to each of 3 guinea-pigs. The mice shall be observed for 7 days and the guinea-pigs for 14 days. None of the animals shall show signs of infection due to the vaccine.

5.5 Test for stability

The vaccine in its final form shall be tested for stability by a method approved by the national control laboratory. The viable

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count of the vaccine shall not fall below \(2 \times 10^9\) live organisms per gelatin capsule throughout its permitted storage period.

5.6 Inspection of final containers

Samples of each final lot shall be inspected visually, and those that show abnormalities shall be discarded.

6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2) 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

As it is not possible to label the gelatin capsules, the package or the leaflet of the capsules shall be labelled according to the requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2).

It should include an indication that inactivation of the vaccine by gastric acid should be prevented by sodium bicarbonate.

9. DISTRIBUTION AND SHIPPING

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2)
10. STORAGE AND EXPIRY DATE

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

10.1 Storage conditions

The manufacturer shall recommend such conditions of storage and shipping as will ensure that the vaccine conforms to the present requirements until the expiry date as stated on the label.

The capsules should be stored in a dry place and in the dark at 5°C ± 3°C.

10.2 Expiry date

The expiry date for the capsules shall be not more than 18 months from the date of manufacture.

If data showing greater stability are available a later expiry date may be permitted.

PART B.

NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substance No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (2) shall apply.
2. RELEASE AND CERTIFICATION

A vaccine lot shall be released only if it fulfils all requirements set forth in Part A of the present requirements.

The national control authority shall ensure that the presentation of the vaccine is such that living Ty 21a bacteria are able to cross the acidic gastric secretions and to infect the intestines.

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 the lot of vaccine in question meets all national requirements as well as Part A of the present requirements. The certificate shall also state the date of the last satisfactory test for viable count, 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 typhoid vaccine between countries.

AUTHORS

The Requirements for Typhoid Vaccine (Live Attenuated, Ty 21a, Oral) were prepared by:
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ACKNOWLEDGEMENTS

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Dr J. Cameron, Director, Biological Products, Institute Armand-Frappier, Laval-des-Rapides, Quebec, Canada
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Dr E.J. Rutenberg, Director, National Institute for Public Health, Bilthoven, Netherlands
Professor G.R.E. Swanker, Brentford, London, England

REFERENCES

Appendix

SUMMARY PROTOCOL OF TYPHOID VACCINE
(LIVE ATTENUATED, Ty 21a, ORAL)

PRODUCTION AND TESTING

Based on Requirements for Biological Substances No. 34,
Requirements for Typhoid Vaccine (Live Attenuated, Ty 21a,

Identification of Final Lot

Name and address of manufacturer

Lot number of final product

Date of manufacture of final lot (namely,
date of initiation by the manufacturer
of the last valid viable count)

Nature of final product (gelatin capsules/
enteric-coated capsules)

Information on Manufacture

As the information on the seed lot will be identical for many batches, the pro-
ducer is advised to make copies of this part of the protocol for future batches.

Working seed lot of S. typhi Ty 21a used

Date of manufacture of parent seed

Date of manufacture of working seed

Number of viable organisms per
ampoule

Tests on working seed

(a) Appearance of growth (Gram stain
and motility)

(b) Antigens when grown without
galactose

Antigens when grown with galactose

(c) Growth on indicator medium

Presence of galactose-fermenting
colonies

65
(d) Colony appearance on indicator medium

(e) Result of test on Kliger iron agar

Galactose-induced bacteriolysis

Mouse virulence

Number of mice injected

Percentage survival

Galactose metabolism enzymes

<table>
<thead>
<tr>
<th>Ty 21a</th>
<th>Ty 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epimerases</td>
<td>+/-</td>
</tr>
<tr>
<td>Galactokinase (EC 2.1.1.6)</td>
<td>...%</td>
</tr>
<tr>
<td>Galactose-1-phosphate-uridylyltransferase (EC 2.1.1.10)</td>
<td>...%</td>
</tr>
<tr>
<td>Galactose-permease</td>
<td>...%</td>
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</tbody>
</table>

Uptake and distribution of $^{14}$C galactose

<table>
<thead>
<tr>
<th>Ty 21a</th>
<th>Ty 2</th>
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</thead>
<tbody>
<tr>
<td>% of galactose taken up after 7 hours</td>
<td>...%</td>
</tr>
<tr>
<td>$^{14}$C galactose remaining in bacteria + medium</td>
<td>...%</td>
</tr>
<tr>
<td>% incorporated in cell wall</td>
<td>...%</td>
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Characterization of LPS

<table>
<thead>
<tr>
<th>Ty 21a</th>
<th>Ty 2</th>
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<tr>
<td>Of cells grown in presence of 1 g/litre $^{14}$C galactose</td>
<td>Smooth ...%</td>
</tr>
<tr>
<td>Rough Ra ...%</td>
<td>Rough Ra ...%</td>
</tr>
<tr>
<td>Rough Re ...%</td>
<td>Rough Re ...%</td>
</tr>
<tr>
<td>Ratio KDO/galactose/glucose/rhamnose</td>
<td></td>
</tr>
</tbody>
</table>

This section of the protocol shall be completed for each final lot.

**Production**

Culture medium used

Temperature and time of incubation

Number of passages to final fermenter

Number of single harvests (fermenters) used in final bulk

66
<table>
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<tr>
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<tbody>
<tr>
<td><strong>1. Identity</strong></td>
</tr>
<tr>
<td>Growth on indicator medium</td>
</tr>
<tr>
<td>Presence of galactose-fermenting colonies</td>
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<tr>
<td>Colony appearance on indicator medium</td>
</tr>
<tr>
<td><strong>2. Viable organisms</strong></td>
</tr>
<tr>
<td>Viable count</td>
</tr>
<tr>
<td>Percentage of survival after freezedrying</td>
</tr>
<tr>
<td><strong>3. Residual moisture</strong></td>
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<tr>
<td>Residual moisture</td>
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</table>

<table>
<thead>
<tr>
<th>Filling into capsules</th>
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</thead>
<tbody>
<tr>
<td>Date of filling</td>
</tr>
<tr>
<td>Filling material</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tests on final product</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Identity</strong></td>
</tr>
<tr>
<td>Colony appearance on indicator medium</td>
</tr>
<tr>
<td><strong>2. Viable count</strong></td>
</tr>
<tr>
<td>Number of viable organisms per capsule</td>
</tr>
<tr>
<td><strong>3. Gross contamination</strong></td>
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<td>Media used</td>
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</table>

<table>
<thead>
<tr>
<th>Pathogens found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of non-pathogenic bacteria per capsule</td>
</tr>
<tr>
<td>Number of fungi per capsule</td>
</tr>
</tbody>
</table>
4. Innocuity
   Number of mice injected
   Quantity injected
   Number of guinea-pigs on test
   Quantity given orally
   Result of tests

5. Result of mouse protection test, if done

6. Visual inspection result

Expiry date
Has the lot been released by the national control authority?
If so, date:
Can a certificate, referred to in the Requirements for Typhoid Vaccine (Live Attenuated, Ty 21a, Oral), be supplied by the national control laboratory?
Which laboratory would supply such a certificate?

Signature:
Name (typed or block letters)
Designation/title
Date

The protocol must be accompanied by a sample of the label and a copy of the leaflet.
Annex 4

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

The list of international biological standards, international biological reference preparations, and international biological reference reagents previously included as annexes to the reports of the WHO Expert Committee on Biological Standardization is issued as a separate publication. The most recent list was published in 1982 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 made the following changes to the 1982 list:

ADDITIONS

Established

Antibiotics

Amikacin 50 600 IU/ampoule  First International Standard 1983

(This substance is held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England).

Antigens

Rabies vaccine 7.8 IU/ampoule  First International Standard 1983

(This substance is held and distributed by the International Laboratory for Biological Standards, State Serum Institute, 80 Amager Boulevard, 2300 Copenhagen, Denmark).

Short ragweed pollen extract 100,000 IU/ampoule First International Standard 1983
Timothy grass pollen extract 100,000 IU/ampoule First International Standard 1983

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

**Blood products and related substances**

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<th>Substance</th>
<th>International Sensitivity Index</th>
<th>Reference Preparation</th>
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<tr>
<td>Thromboplastin, bovine, combined</td>
<td>1.0</td>
<td>Second International Reference Preparation 1983</td>
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<td>Thromboplastin, human, plain</td>
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<td>Second International Reference Preparation 1983</td>
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</table>

(These substances are held and distributed by the International Laboratory for Biological Standards, Central Laboratory, Red Cross Blood Transfusion Service, Plesmanlaan 125, Amsterdam, Netherlands).

Heparin 1780 IU/ampoule Fourth International Standard 1983

(This preparation is held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England).

**Endocrinological and related substances**

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<thead>
<tr>
<th>Substance</th>
<th>IU/ampoule</th>
<th>Reference Preparation</th>
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</thead>
<tbody>
<tr>
<td>Thyroid stimulating hormone, human, for immunoassay</td>
<td>0.037</td>
<td>Second International Reference Preparation 1983</td>
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</tbody>
</table>

(This substance is held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England).
Miscellaneous substances

Rubella virus
(strain M-33)  International
Rubella antiserum,
rabbit  Reference
                  Reagent 1983

(These reagents are held and distributed by the International Laboratory for Biological Standards, State Serum Institute, 80 Amager Boulevard, 2300 Copenhagen, Denmark).

Human anti-nuclear
ribonucleoprotein
serum  International

Human anti-smooth
muscle serum  Reference
                  Reagent 1983

(These reagents are held and distributed by the International Laboratory for Biological Standards, Central Laboratory, Red Cross Blood Transfusion Service, Plesmanlaan 125, Amsterdam, Netherlands).

DISCONTINUED

Antigens

Lecithin (bovine
heart)  Second International Reference
                  Preparation
                  1953

Blood products and related substances

Thromboplastin,
human, combined  First International Reference
                  Preparation
                  1976
Annex 5

REQUIREMENTS FOR BIOLOGICAL SUBSTANCES
AND OTHER SETS OF RECOMMENDATIONS

The specification of requirements to be fulfilled by preparations of biological substances is necessary in order to ensure that these products are safe, reliable, and potent prophylactic or therapeutic agents. International recommendations on requirements are intended to facilitate the exchange of biological substances between 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 Technical Report Series of the World Health Organization are listed hereunder:

<table>
<thead>
<tr>
<th>No.</th>
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<th>Requirements for Biological Substances:</th>
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<tbody>
<tr>
<td>178</td>
<td>1959</td>
<td>* 1. General Requirements for Manufacturing Establishments and Control Laboratories</td>
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<tr>
<td></td>
<td></td>
<td>* 2. Requirements for Poliomyelitis Vaccine (Inactivated)</td>
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<td>179</td>
<td>1959</td>
<td>* 3. Requirements for Yellow Fever Vaccine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 4. Requirements for Cholera Vaccine</td>
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<tr>
<td>180</td>
<td>1959</td>
<td>* 5. Requirements for Smallpox Vaccine</td>
</tr>
<tr>
<td>200</td>
<td>1960</td>
<td>* 6. General Requirements for the Sterility of Biological Substances</td>
</tr>
<tr>
<td>237</td>
<td>1962</td>
<td>* 7. Requirements for Poliomyelitis Vaccine (Oral)</td>
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<tr>
<td>274</td>
<td>1964</td>
<td>* 8. Requirements for Pertussis Vaccine</td>
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<td></td>
<td></td>
<td>* 9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate</td>
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<td>293</td>
<td>1964</td>
<td>* 10. Requirements for Diphtheria Toxoid and Tetanus Toxoid</td>
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* Replaced by revised Requirements.
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<tr>
<th>No.</th>
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<tr>
<td>323</td>
<td>1966 WHO Expert Group: Requirements for Biological Substances (Revised 1965):</td>
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<tr>
<td></td>
<td>1. General Requirements for Manufacturing Establishments and Control Laboratories</td>
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<tr>
<td></td>
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<td>5. Requirements for Smallpox Vaccine</td>
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<td></td>
<td>* 7. Requirements for Poliomyelitis Vaccine (Oral)</td>
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<td>329</td>
<td>1966 WHO Expert Committee on Biological Standardization:</td>
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<td></td>
<td>* 11. Requirements for Dried BCG Vaccine</td>
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<td></td>
<td>12. Requirements for Measles Vaccine (Live) and Measles Vaccine (Inactivated)</td>
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<tr>
<td>361</td>
<td>1967 WHO Expert Committee on Biological Standardization:</td>
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<tr>
<td></td>
<td>9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate (Revisions adopted 1966)</td>
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<td></td>
<td>13. Requirements for Anthrax Spore Vaccine (Live—for Veterinary Use)</td>
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<td>14. Requirements for Human Immunoglobulin</td>
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<td>15. Requirements for Typhoid Vaccine</td>
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<td>384</td>
<td>1968 WHO Expert Committee on Biological Standardization:</td>
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<td></td>
<td>16. Requirements for Tuberculins</td>
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<td></td>
<td>* 17. Requirements for Inactivated Influenza Vaccine</td>
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<tr>
<td>413</td>
<td>1969 WHO Expert Committee on Biological Standardization:</td>
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<td></td>
<td>† 4. Requirements for Cholera Vaccine (Revised 1968)</td>
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<td>18. Requirements for Immune Sera of Animal Origin</td>
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<td>444</td>
<td>1970 WHO Expert Committee on Biological Standardization:</td>
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<td></td>
<td>19. Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)</td>
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<td>† 20. Requirements for <em>Brucella abortus</em> Strain 19 Vaccine (Live—for Veterinary Use)</td>
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<td>444</td>
<td>1970 WHO Expert Committee on Biological Standardization:</td>
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<td></td>
<td>* Development of a National Control Laboratory for Biological Substances (A guide to the provision of technical facilities)</td>
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<td>463</td>
<td>1971 WHO Expert Committee on Biological Standardization:</td>
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<td>21. Requirements for Snake Antivenins</td>
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<td>486</td>
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<td>* 7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1971)</td>
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<td>530</td>
<td>1973 WHO Expert Committee on Biological Standardization:</td>
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<td>4. Requirements for Cholera Vaccine (Revised 1968) (Addendum 1975)</td>
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* Replaced by revised Requirements.
† Refer also to subsequent Addendum.
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 Immunoassay and Receptor Assay Systems) for Human Hormones and their Binding Proteins
(A guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytochemical bioassay systems)
Development of national assay services for hormones and other substances in community health care

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

610 1977 WHO Expert Committee on Biological Standardization:

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)
† 26. Requirements for Antibiotic Susceptibility Tests. I. Agar Diffusion Tests using Antibiotic Susceptibility Discs

626 1978 WHO Expert Committee on Biological Standardization:
17. Requirements for Inactivated Influenza Vaccine (Addendum 1977)
† 23. Requirements for Meningococcal Polysaccharide Vaccine (Addendum 1977, incorporating Addendum 1976)
27. Requirements for the Collection, Processing, and Quality Control of Human Blood and Blood Products

626 1978 WHO Expert Committee on Biological Standardization:
Guidelines for the Preparation and Establishment of Reference Materials for Biological Substances

638 1979 WHO Expert Committee on Biological Standardization:
† 8 and 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Revised 1978)

* Replaced by revised Requirements.
† Refer also to subsequent Addendum.
<table>
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<th>Year of Publication</th>
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<td>† 8 and 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Addendum 1980)</td>
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<td>22. Requirements for Rabies Vaccine for Human Use (Revised 1980)</td>
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<td>23. Requirements for Meningococcal Polysaccharide Vaccine (Addendum 1980)</td>
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<td>29. Requirements for Rabies Vaccine for Veterinary Use</td>
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<td>31. Requirements for Hepatitis B Vaccine</td>
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<td>* 26. Requirements for Antibiotic Susceptibility Tests (Suggested changes 1980)</td>
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<td>24. Requirements for Rubella Vaccine (Live) (Addendum 1980)</td>
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<td>* 30. Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy</td>
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<td>Guidelines for Quality Assessment of Antimicrobial Agents</td>
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<td></td>
<td></td>
<td>The National Control of Vaccines and Sera</td>
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<td>Requirements for Immunoassay Kits</td>
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<tr>
<td>673</td>
<td>1982</td>
<td>2. Requirements for Poliomyelitis Vaccines (Inactivated) (Revised 1981)</td>
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<td>8 and 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Addendum 1981)</td>
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<td></td>
<td>† 26. Requirements for Antimicrobial Susceptibility Tests (Revised 1981)</td>
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<tr>
<td></td>
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<td>32. Requirements for Rift Valley Fever Vaccine</td>
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<tr>
<td></td>
<td></td>
<td>A Review of Tests on Virus Vaccines</td>
</tr>
<tr>
<td>687</td>
<td>1983</td>
<td>WHO Expert Committee on Biological Standardization:</td>
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<td>7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1982)</td>
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<td>30. Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy (Revised 1982)</td>
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<td>33. Requirements for Louse-Borne Human Typhus Vaccine (Live)</td>
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<td>The Standardization of Interferons</td>
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<td>26. Requirements for Antimicrobial Susceptibility Tests (Addendum 1982)</td>
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<td>35. Requirements for Rift Valley Fever Vaccine (Live, Attenuated for Veterinary Use)</td>
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<td>34. Requirements for Typhoid Vaccine (Live Attenuated, Ty 21a, Oral)</td>
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* Replaced by revised Requirements.  
† Refer also to subsequent Addendum.
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<td>675</td>
<td>Chemotherapy of leprosy for control programmes</td>
<td>33</td>
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