World Health Organization, Technical Report Series, No. 800, 1990

Annex 2

REQUIREMENTS FOR DIPHTHERIA, TETANUS, PERTUSSIS AND COMBINED VACCINES

(Requirements for Biological Substances Nos. 8 and 10) (Revised 1989)

INTRODUCTION

The WHO Requirements for Pertussis (P) Vaccine were formulated in 1963 (*1*) (Requirements for Biological Substances

No. 8) and those for Diphtheria (D) and Tetanus (T) Toxoid Vaccines in 1964 (*2*) (Requirements for Biological Substances No. 10); the Requirements for the three vaccines (DPT) were revised in 1978 (*3*) (Requirements for Biological Substances Nos. 8 and 10). Diphtheria and tetanus toxoids and pertussis vaccine are most commonly used in a combined form, and the 1978 revision covered all three vaccines in a single document.

At the end of May 1988, WHO organized a three-day scientific consultation in Geneva during which the changes in the production and control methods for diphtheria, pertussis and tetanus vaccines that had occurred over the previous 10 years were reviewed (*4*). Special emphasis was placed on methods of determining the potency of diphtheria and tetanus (DT) toxoid vaccines that would require a smaller number of animals. While methods for determining the antigen content of DT vaccines have been available for many years, their results do not necessarily indicate whether the vaccines are of adequate potency. This is not only because antigens that cannot be eluted from adjuvants may not be determined by these methods but also because they do not take into account certain important characteristics of vaccines, such as those associated with the conditions of inactivation, purification and adsorption, which may influence protective capacities. Immunogenicity tests in animals are therefore necessary for assessing the potency of toxoid vaccines. There are, however, different opinions on the way such tests should be conducted; in some countries, tests are carried out in such a way that potencies cannot be expressed in International Units of toxoid.

These revised Requirements were prepared by a group of experts and staff members (see p. 149) who met immediately after the 1988 consultation and reviewed the regulations for D, T, P and combined vaccines in force in a number of countries. In addition, comments have been received from a number of experts, whose assistance is gratefully acknowledged (see p. 150).

The main changes made to the 1978 revision are as follows:

(1) The term "toxoid" is replaced where appropriate by "vaccine" and the order in which pertussis and tetanus vaccines are dealt with has been reversed.
(2) Greater flexibility is allowed in the conduct of sterility tests so as to permit the use of satisfactory methods adopted by national control authorities.

(3) Attention is drawn to the need to ensure that the purification methods used exclude from final products substances derived from the medium that are likely to have sensitizing properties.
(4) The need to ensure that vaccines still possess the minimum potency at the expiry date is emphasized in a paragraph in the sections on final products; this requires the determination of the loss of potency during storage at the recommended temperature and the successful testing of three consecutive batches of vaccines.

Other changes in the requirements not common to the three vaccines are mentioned in the general considerations for each product.

In a more general way, the experts who prepared these Requirements agreed that methods of production and testing other than those described in them should be acceptable provided that they have been properly validated and that they yield products as safe and protective as those prepared using the methods described here.

Diphtheria, tetanus and pertussis vaccines are often used in combination and usually contain an adjuvant. The formulations commonly used include:

- tetanus vaccine adsorbed,
- diphtheria and tetanus vaccine adsorbed,
- tetanus and diphtheria vaccine adsorbed for adult use (Td),
- pertussis vaccine adsorbed,
- diphtheria, tetanus and pertussis vaccine adsorbed.

Plain tetanus vaccine is sometimes used for primary immunization but there is no agreed potency expressed in International Units for this type of preparation. The potency of such vaccines should therefore be approved by the national control authority.

The requirements for diphtheria vaccine, tetanus vaccine, pertussis vaccine and combined vaccines (DT and DTP) are dealt with in separate sections. As in the previous Requirements, the separate section on combined vaccines has been included since certain special tests are applicable only to such formulations.

REQUIREMENTS FOR DIPHTHERIA VACCINE (ADSORBED)

GENERAL CONSIDERATIONS

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

The early developments leading to the formulation of the first Requirements for Diphtheria Toxoid are described in detail in the Requirements for Biological Substances No. 10 (*2*).

The Parke Williams 8 strain of *Corynebacterium diphtheriae* has been shown to be satisfactory for producing potent diphtheria vaccines, and no purpose would seem to be served by suggesting a change of strain. The approach adopted in diphtheria vaccine production is to obtain the greatest possible quantity of toxin during the growth phase of the microorganisms and thereafter to convert the toxin into stable toxoid by the most efficient method.

One of the most important achievements in drawing up the first Requirements for Diphtheria Toxoid was the agreement reached on the formulation of requirements for the assay of potency. In 1964, almost all countries had adopted their own requirements and there were considerable differences between them. By 1978, a number of countries had adopted requirements based on a comparison of the protection afforded to laboratory animals by graded doses of the test vaccine and that provided by a reference vaccine that had been calibrated in International Units. A requirement involving the immunization of guinea-pigs followed either by a lethal challenge test or an intradermal challenge test in which graded doses of toxin are administered was therefore included in the 1978 revision of the Requirements. The former indicated that the immunized animals were protected against a single challenge dose of toxin, whereas the latter test, by virtue of the graded doses of challenge toxin used, gave a quantitative measure of the animals' level of immunity.

Following the publication of the 1978 revised Requirements, it became apparent that the large numbers of animals, particularly of guinea-pigs, required for the potency test made conformity difficult

to achieve in many countries. Means of reducing the number of animals required, without prejudice to the principle of expressing potency in terms of International Units, have therefore been sought, the emphasis being on the use of the minimum number of animals necessary to provide assurance that the potency of the vaccine is indeed greater than the minimum required. A step was taken in that direction in 1986 (*5*) when an addendum to Requirements Nos. 8 and 10 specified that ranges of 95% confidence intervals greater than 50–200% were acceptable provided that the lower limit of the 95% confidence interval was still above the minimum potency required in each single human dose.

A further method of reducing the number of animals used in three-dilution assay systems is to determine the individual titres of antitoxin of laboratory animals such as mice or guinea-pigs by toxin neutralization tests in cell cultures. Further development of a variety of methods specific for the assay of diphtheria antitoxin should also result in a reduction in the number of laboratory animals used.

The number of animals used in tests based on challenge can also be reduced by assaying both the test and reference vaccine at a single dilution, provided that the test is performed by laboratories with extensive experience of vaccines on which three-dilution assays have been regularly and sucessfully performed.

Although the available data are insufficient to permit a correlation between a potency level observed in a laboratory assay and protection in humans or the duration of immunity, some evidence is available from which a potency level can be specified above which a vaccine may be considered to be of acceptable potency. It is important, therefore, for the potency of diphtheria vaccines to be expressed in International Units. In some countries, potency tests based on the ability of the vaccine to induce specified levels of antitoxin are used, but such assays are often performed on pooled sera and without the use of calibrated reference materials, and do not allow a valid statistical evaluation of the results to be made. Although data are available that demonstrate that vaccines meeting such requirements can induce significant levels of antitoxin response in humans, the use of more quantitative types of assays is recommended in these revised Requirements for Diphtheria Vaccine (Adsorbed).

In addition, whenever new vaccines are produced, long-term studies should be carried out to confirm that, in 90% of the target population, diphtheria antitoxin levels are above 0.01 IU/ml five

years after the completion of primary immunization in previously unvaccinated individuals.[1]

These Requirements call for the product to be purified, since diphtheria toxoid in the unpurified form is liable to give rise to severe vaccination reactions in humans; much work has therefore been done in developing purified material in order to avoid them. Even with purified products, however, untoward reactions may occur in adults. In view of the risk of reversion to toxicity, especially when a toxin is detoxified after purification, the Requirements have been formulated so as to exclude this risk and, in the irreversibility test, the incubation period at 37 °C of the purified toxoid has been increased to six weeks. There is evidence that purification may sometimes reduce the immunizing activity of diphtheria toxoid, probably as a result of the removal of substances having an adjuvant effect. Purified products, if intended for primary immunization, must therefore be combined with a mineral adjuvant, although they may be used uncombined for reinforcing immunity. In these revised Requirements, the maximum number of "limit of flocculation" or Lf units per single human dose of diphtheria vaccine (adsorbed) has been reduced to 30. These requirements do not apply to plain diphtheria vaccines, although such vaccines are sometimes used for primary immunization.

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

Individual countries may wish to adopt these Requirements as the basis of their national regulations on diphtheria vaccines. If national requirements differ from these requirements, it is recommended that the former should be shown to ensure that the vaccine is at least as safe and as potent as that prepared in accordance with the requirements formulated below. It is desirable that the World Health Organization should be kept informed of any such differences.

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[1] Primary immunization usually consists of an initial course of two or three injections at intervals of 4–6 weeks, followed by a further injection 7–12 months later. Immunity can be reinforced or "boosted" by subsequent single injections, usually given a number of years later.

PART A. MANUFACTURING REQUIREMENTS

A.1 Definitions

A.1.1 International name and proper name

The international name shall be *Vaccinum diphtheriae adsorbatum*. The proper name shall be the equivalent of the international name in the language of the country of use.

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

A.1.2 Descriptive definition

Vaccinum diphtheriae adsorbatum is a preparation of diphtheria toxoid prepared by treating diphtheria toxin by chemical means so as to render it nontoxic without destroying its immunogenic potency. The toxoid is adsorbed on to a suitable adjuvant. The preparation shall satisfy the requirements formulated below.

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

A.1.3 International reference materials

The first International Reference Reagent of Diphtheria Toxoid for Flocculation Tests was established in 1988 (*6*).

The second International Standard of Diphtheria Toxoid, Adsorbed, was established in 1978 (*3*) for determining the potencies of vaccines containing adsorbed diphtheria toxoid.

The International Standard for Diphtheria Antitoxin was established in 1934; it is made from horse hyperimmune serum.

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

A.1.4 Terminology

Seed lot: A quantity of bacterial suspension that is derived from one strain, has been processed as a single lot and has a uniform

composition. It is used for preparing the inoculum for the production medium.

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

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

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

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

A.2 General manufacturing requirements

The general requirements for manufacturing establishments contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply to establishments manufacturing diphtheria vaccine with the addition of the following:

Written descriptions of procedures for the preparation and testing of diphtheria vaccine adopted by a manufacturer together with appropriate evidence that each production step has been validated shall be submitted for approval to the national control authority. Proposals for modifications of the manufacturing and/or control methods shall also be submitted for approval to the national control authority before such modifications are implemented.

A.3 Production control

A.3.1 Control of source materials

A.3.1.1 *Strains of Corynebacterium diphtheriae*

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

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

A.3.1.2 *Seed lot system*

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

A.3.1.3 *Culture medium for production of toxin*

It is particularly important to ensure that the final product is free from substances likely to cause toxic or allergic reactions in humans.

The method of detecting these substances should be approved by the national control authority.

If the medium is prepared from a protein digest, e.g., casein hydrolysate or digested muscle, precautions should be taken to ensure that digestion has proceeded sufficiently. Established limits, if any, for mammalian protein and human blood-group substances in the final vaccine should not be exceeded.

A.3.2 Production precautions

The general production precautions, as formulated in Part A, section 3, of Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*), shall apply to the manufacture of diphtheria vaccine.

Suitable methods for the production of diphtheria vaccine are given in the *Manual for the production and control of vaccines: diphtheria toxoid* (*8*).

Personnel employed in production and quality control shall be adequately trained and immunized.

A.3.3 Control of single harvests

Consistency of production shall be demonstrated.

Consistency may be demonstrated by measuring, e.g., the bacterial growth rate, pH and rate of toxin production.

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

A.3.3.1 *Control of bacterial purity*

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

A.3.3.2 *Filtration*

After having been sampled for the control of purity, cultures shall be sterilized by means of filtration. A preservative may be added, but phenol shall not be used for this purpose.

Cultures should be filtered as soon as possible after the end of their incubation period. To facilitate filtration, cultures may be centrifuged, provided that suitable precautions are taken to avoid the formation of potentially hazardous aerosols. A filter aid may be added beforehand.

In some countries, no filter capable of shedding fibres may be used.

A.3.3.3 *Determination of antigen concentration*

The supernatant of the culture prior to inactivation shall be tested by a method approved by the national control authority.

It is advisable to determine the antigen content by measuring the toxin content. This is usually done *in vivo;* however, *in vitro* methods are acceptable if validated.

Another suitable method for determining the antigen concentration is the flocculation test which is described in the *Manual for the production and control of vaccines: diphtheria toxoid* (*8*); it should be performed on both the supernatant and, for purposes of comparison, a reference material calibrated against the International Reference Reagent of Diphtheria Toxoid for Flocculation Tests, or an equivalent reference preparation approved by the national control authority.

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

Antigen content is a good indicator of consistency of production.

A.3.3.4 *Detoxification and purification of toxin*

Purification may either precede or follow detoxification. Purification before detoxification results in a purer product, but particular care must be taken to avoid reversion to toxin, which may also occur when detoxification precedes purification. The method and agent used for detoxification and the method of purification shall be approved by the national control authority.

Amino acids such as lysine are frequently added during detoxification and help to prevent reversion.

After detoxification has been completed, the detoxifying agent shall be removed or neutralized by a method approved by the national control authority.

The method of purification shall be such that no substances are incorporated into the final product that are likely to cause untoward reactions in humans. The rate of detoxification may vary and shall be monitored. Harvests shall not be transferred from the detoxification area until the detoxification has been shown to be complete.

A.3.4 Control of bulk purified toxoid

A.3.4.1 *Preparation*

The bulk purified toxoid shall be prepared from either a single harvest or a pool of single harvests, and shall be sterile. Phenol shall not be used as a preservative.

It is advisable to sterilize the bulk purified toxoid by filtration. A preservative approved by the national control authority may be added to the bulk toxoid.

A.3.4.2 *Sterility*

Each bulk purified toxoid shall be tested for bacterial and mycotic sterility in accordance with the requirements of Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (*9*) or by a method approved by the national control authority. If a preservative has been added to the purified bulk, appropriate measures shall be taken to prevent any interference by it in the sterility test.

A.3.4.3 *Specific toxicity*

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

> Some manufacturers carry out, in addition, a test for determining whether diphtheria toxin is present by injecting intradermally into rabbits or guinea-pigs at least 20 Lf of purified toxoid and observing the injection sites for specific erythema.

Alternatively, a cell-culture test system may be used; in this case, the sensitivity of the test shall have been demonstrated to be not less than that of the guinea-pig test, and the test procedures shall be approved by the national control authority.

A.3.4.4 *Reversion to toxicity*

Each bulk purified toxoid shall be tested to ensure that reversion to toxicity cannot take place on storage. The bulk purified toxoid shall be diluted in order to obtain the same concentration and chemical environment as those present in the final bulk vaccine, except for the presence of adjuvant.

To determine whether reversion has occurred, diluted toxoids that have been stored at 37 °C for six weeks shall be tested. The test employed shall be approved by the national control authority and should be sufficiently sensitive to detect very small amounts of toxin. No toxicity shall be detected.

> In one country, the test is performed on toxoids that have been stored at 34 °C.
>
> Similar dilutions of toxoid held at 2–8 °C during the same period of time as those held at 34 °C or 37 °C may be tested as controls.
>
> Intradermal tests in guinea-pigs and cell-culture tests are both considered to be suitable.

A.3.4.5 *Antigenic purity*

Each bulk purified toxoid shall be tested for antigenic purity by determining the antigen concentration in Lf units and the concentration of protein (nondialysable) nitrogen. The antigen concentration shall be determined by comparison with a reference material calibrated against the International Reference Reagent of Diphtheria Toxoid for Flocculation Tests, or an equivalent reference preparation approved by the national control authority. The method of testing shall be approved by the national control authority. The bulk purified toxoid shall pass the test if it contains no fewer than 1500 Lf per mg of protein (nondialysable) nitrogen.

Preparation of toxoid containing more than 1500 Lf per mg is both feasible and desirable.

An indication of the antigenic quality of the toxoid may be obtained by measuring the total combining power and expressing it in relation to the number of Lf units. A suitable method for measuring the total combining power is given in the *Manual for the production and control of vaccines: diphtheria toxoid* (*8*).

A.3.5 Control of final bulk

A.3.5.1 *Preparation*

The final bulk shall be prepared from bulk purified toxoid. The number of Lf in a single human dose shall be approved by the national control authority but shall not exceed 30.

A.3.5.2 *Preservative*

If the vaccine is to be dispensed into multidose containers, a suitable antimicrobial preservative shall be added. The amount of preservative in the final bulk shall have been shown to have no deleterious effect on the toxoid or on other vaccine components with which the toxoid may be combined, and to cause no unexpected adverse reactions in humans. The preservative and its concentration shall be approved by the national control authority.

Phenol shall not be used as a preservative.

A.3.5.3 *Adjuvants*

The adjuvants used, their purity and their concentration shall be approved by the national control authority.

Aluminium or calcium compounds are generally used as mineral carriers.

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

In some countries, these upper limits for the concentrations of mineral carriers are considered to be too high and the limits are set at about half those given above.

In some countries, the adsorbent is precipitated in the presence of the toxoid.

The formulation shall be such that the vaccine remains suspended for a reasonable time after shaking.

A.3.5.4 *Sterility*

Each final bulk shall be tested for bacterial and mycotic sterility in accordance with the requirements of Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (*9*) or by a method approved by the national control authority. If a preservative has been added to the final bulk, appropriate measures shall be taken to prevent any interference by it in the sterility test.

A.3.5.5 *Specific toxicity*

In some countries, each final bulk is tested for specific toxicity in at least five guinea-pigs, each weighing 250–350 g. Each guinea-pig is given a subcutaneous injection of a quantity equivalent to at least five single human doses. Animals that die are autopsied and examined for symptoms of diphtheria intoxication (red adrenals). The final bulk passes the test if no guinea-pig shows symptoms of specific intoxication within six weeks of injection and if at least 80% of the animals survive the test period. The guinea-pigs must not have been used previously for experimental purposes.

A.3.5.6 *Potency*

The immunizing potency of each final bulk shall be determined by comparison with an appropriate reference material calibrated against the International Standard for Diphtheria Toxoid, Adsorbed. The determination shall involve the inoculation of guinea-pigs or mice with appropriate doses or dilutions of both the product and the reference material. After immunization, mice shall

be bled, and guinea-pigs bled or challenged either by the subcutaneous or by the intradermal route (*10*). When animals are bled, the antitoxin levels of the individual animals may be titrated by means of toxin neutralization tests performed using *in vivo* or *in vitro* serological methods[1] that have been validated on vaccines of the types being tested. Appropriate statistical methods shall be used to calculate the potency of the final bulk (*10*). The method adopted and the interpretation of the results shall be approved by the national control authority.

Care should be taken to ensure that diluents are inert and not pyrogenic. Phosphates might interfere with the adsorption of toxoid.

When consistency of production and testing have been established, the numbers of animals injected with each dilution of product may be reduced to levels substantially lower than those originally needed for the three-dilution assays described in the *Manual of details of tests required on final vaccines used in the WHO Expanded Programme on Immunization* (*10*), provided that the resulting assays are statistically valid. Methods based on individual quantification of antitoxin or, in the case of guinea-pigs, scores of responses to intradermal challenge, allow the use of fewer animals than are needed in lethal challenge tests.

Depending on the purpose, two types of potency assays may be considered.

Three-dilution assays may be used to test consistency of production and product stability, and to calibrate reference preparations.

One-dilution assays,[2] based on the same principles for evaluating the response as the three-dilution assays, may be used at the discretion of the national control authority for the routine testing of vaccine lots of a given formulation as soon as the production process has been established and consistency in production and control has been demonstrated. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 30 IU (i.e., of the minimum potency of a single human dose), that elicits a minimal protective effect in guinea-pigs, and comparing its effect with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the latter is significantly greater than that to

[1] Information on potency determination of the diphtheria component of D, DT and DTP vaccines in mice based on antitoxin assay by toxin neutralization in Vero cell cultures is given in unpublished WHO document BS/89.1613, which can be obtained from Biologicals, World Health Organization, Geneva, Switzerland.

[2] Information on one-dilution assay methods is given in document BS/89.1618, available on request from Biologicals, World Health Organization, Geneva, Switzerland.

the former ($P \leqslant 0.05$), the potency of the test vaccine is satisfactory. One-dilution tests offer advantages only when vaccine potencies are consistently and substantially in excess of 30 IU per single human dose.

The potency of the final bulk shall be approved by the national control authority. The potency of diphtheria vaccine used for the immunization of children shall not be less than 30 IU per single human dose. For three-dilution assays, the limits of the 95% confidence intervals of the estimate of potency shall be within 50–200% of the estimated potency unless the lower limit of the 95% confidence interval of the estimated potency is greater than 30 IU per single human dose. When one-dilution tests are performed, the potency of the test vaccine shall be demonstrated to be significantly greater than 30 IU per human dose.

In some countries, vaccines intended for the booster immunization of adults contain less than 30 IU per dose.

In some countries, potency testing is not carried out on each final bulk but on each final lot.

A.3.5.7 *Amount of residual free detoxifying agent*

The amount of residual free detoxifying agent in each final bulk shall be determined by a method approved by the national control authority, and, if formaldehyde has been used, the residual content shall be not more than 0.2 g/l.

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

In some countries, the amount of residual free detoxifying agent is determined in the purified bulk.

If applicable, appropriate tests for the presence of other detoxifying agents (e.g., glutaraldehyde) shall be performed. The tests used and the maximum permissible concentrations of such chemicals shall be approved by the national control authority.

A.3.5.8 *pH*

The pH of the final bulk shall be measured.

The pH should be between 6.0 and 7.0.

A.4 Filling and containers

The requirements applicable to filling and containers given in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply.

Single-dose or multiple-dose containers may be used. Vaccine in multidose containers shall contain a suitable antimicrobial preservative.

A.5 Control of final product

A.5.1 Identity

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

> Flocculation in solution, immunoprecipitation of the toxoid in gels or any other specific interaction between the vaccine and diphtheria antitoxin may serve as an identity test. Tests on toxoids adsorbed on to aluminium or calcium carrier may be performed after the carrier has been dissolved, or the adsorbed toxoid wholly or partially eluted by sodium citrate at pH 9.
>
> If adequate quantities of toxoid cannot be recovered from the adsorbed vaccine, specific antitoxin may be sought in the sera of animals used in the innocuity test.

A.5.2 Sterility

Final containers shall be tested for bacterial and mycotic sterility by a method approved by the national control authority.

Many countries have regulations governing the sterility testing of the final product. Where these do not exist, the requirements published by WHO shall be met (*9*). If a preservative has been added to the purified bulk, appropriate measures shall be taken to prevent any interference by it in the sterility test.

A.5.3 Potency

A potency test shall be carried out, as provided in Part A, section A.3.5.6, on each final lot, if such a test has not been performed on the final bulk.

A.5.4 Innocuity

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

A.5.5 Adjuvant content

The adjuvant content of each final lot shall be determined by a method approved by the national control authority (see Part A, section A.3.5.3).

In some countries, this test is used to verify the homogeneity of filling.

A.5.6 Preservative content

The preservative content of each final lot shall be determined (see Part A, section A.3.5.2). The method used shall be approved by the national control authority.

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

A.5.7 pH

The pH of each final lot shall be measured.

The pH should be between 6.0 and 7.0.

A.5.8 Inspection of final containers

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

A.6 Records

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

ments for Manufacturing Establishments and Control Laboratories) (*7*) shall apply.

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

A model of a suitable summary protocol to be used for diphtheria vaccines is given in Appendix 1.

A.7 Samples

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

A.8 Labelling

The label printed on or affixed to each container and the label on the carton enclosing one or more containers shall show as a minimum:

- the words *Vaccinum diphtheriae adsorbatum* and/or the proper name of the product,
- the name and address of the manufacturer,
- the number of the final lot,
- the recommended storage temperature and the expiry date if kept at that temperature, and
- the recommended single human dose and route of administration.

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

- a statement that the vaccine satisfies the requirements of this document,
- the nature and amount of any preservative present in the vaccine,
- the nature and amount of the adsorbing agent,
- the recommended temperature for storage and transport,
- a warning that the adsorbed vaccine should not be frozen,
- a warning that the adsorbed vaccine should be shaken before use, and

- instructions for the use of the vaccine and information on contraindications and the reactions that may follow vaccination.

A.9 Distribution and transport

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply.

A.10 Stability, storage and expiry date

A.10.1 Stability

Tests shall be conducted to determine the loss of potency to be expected during storage. The stability of the vaccine shall be demonstrated to the satisfaction of the national control authority; final containers from at least three lots derived from different lots of purified bulk toxoid shall be tested on the expiry date to demonstrate stability during storage. The vaccine shall meet the requirements for final product (see Part A, sections A.5.3, A.5.4, A.5.7 and A.5.8) up to the expiry date, provided that it has been stored at the recommended temperature. When any changes are made in the production procedure that may affect the stability of the product, the vaccine produced by the new method shall be shown to be stable.

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

A.10.2 Storage conditions

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

Adsorbed vaccines shall not be frozen.

A.10.3 Expiry date

The expiry date shall be approved by the national control authority based on the stability studies referred to in section A.10.1

and shall relate to the date of the last satisfactory potency determination, performed in accordance with Part A, section A.5.3, i.e., the date on which the test animals were immunized with the vaccine.

PART B. NATIONAL CONTROL REQUIREMENTS

B.1 General

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply.

The detailed production and control procedures and any significant changes in them shall be discussed with, and approved by, the national control authority, which shall obtain the International Standard for Diphtheria Toxoid, Adsorbed and establish a national working reference preparation by comparison with it.

B.2 Official release and certification by the national control authority

A vaccine shall be released only if it satisfies Part A of the present Requirements.

A statement signed by the appropriate official of the national control authority shall be provided at the request of the manufacturing establishment and shall certify that the lot of vaccine in question satisfies all national requirements as well as Part A of the present Requirements. The certificate shall state the number under which the lot was released by the national control authority, and the number appearing on the labels of the containers. The official national release document shall be provided to importers of diphtheria vaccines.

> The purpose of the certificate is to facilitate the exchange of diphtheria vaccines between countries. A model of a suitable certificate is given in Appendix 2.

REQUIREMENTS FOR TETANUS VACCINE (ADSORBED)

GENERAL CONSIDERATIONS

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

The developments leading to the formulation of the first Requirements for Tetanus Toxoid are described in detail in the Requirements for Biological Substances No. 10 (*2*). The purpose of the present General Considerations section is to draw attention to the significant developments that have taken place since those Requirements were revised in 1978 (*3*).

As with diphtheria vaccine, the most important development is the agreement reached on the formulation of requirements for the assay of potency. It has now been accepted that the potency of tetanus vaccine can be measured by an active challenge test and that either guinea-pigs or mice may be used. In studies of the use of a lethal challenge dose as compared with a paralytic challenge dose, it was found that they give similar results when the potency of a test vaccine is compared with that of a reference preparation. It is important to note, however, that when pertussis vaccine is mixed with tetanus toxoid and when the potency assay is carried out in mice, there is a significant adjuvant effect due to the whole-cell pertussis component. Allowance must be made for this effect in the assay of a combined vaccine to determine the potency of the tetanus component. The minimum acceptable level of potency expressed in International Units must be specified by the national control authority.

The statements made in the section on diphtheria vaccines dealing with the reduction in the number of laboratory animals used in potency tests based on challenge (see pp. 91–92) are equally applicable to tetanus vaccines. Further development of a variety of specific methods for the assay of tetanus antitoxin should also result in a reduction in the utilization of laboratory animals.

Although there are few data on which a correlation between the potency level determined by a biological assay and protection in humans could be based, and even fewer on which to base a correlation between the potency level and the duration of immunity, some evidence is available that makes it possible to specify a level above which a vaccine may be considered to be of acceptable potency. Such a level is included in the present Requirements. It is important, therefore, for countries to adopt the principle of expressing the potency of tetanus vaccines in International Units. In some countries, potency tests based on the ability of vaccines to induce specified levels of antitoxin are used, but such assays are often performed on pooled sera and without the use of calibrated reference materials, so that a valid statistical evaluation of the results cannot be made. Although data are available that demonstrate that vaccines meeting such requirements can induce significant levels of antitoxin response in humans, the use of more quantitative types of assays is recommended in these revised Requirements for Tetanus Vaccine (Adsorbed).

In addition, it is desirable that, whenever new vaccines are produced, long-term studies are carried out to confirm that, in 90% of the target population, tetanus antitoxin levels are above 0.01 IU/ml five years after the completion of primary immunization in previously unimmunized individuals.[1]

These Requirements call for the product to be purified, since tetanus toxoid in the unpurified form is liable to give rise to vaccination reactions in humans; much work has therefore been done in developing purified material in order to avoid them. Even with purified products, however, untoward reactions may occur in adults. There is evidence that purification, although enabling more highly concentrated preparations to be used, may sometimes reduce the immunizing activity of tetanus vaccine, probably as a result of the removal of substances having an adjuvant effect. Such purified products may be used for primary immunization after combination with an adjuvant. In these revised Requirements, the maximum number of Lf per single human dose of tetanus vaccine (adsorbed)

[1] Primary immunization usually consists of an initial course of two or three injections at an interval of 4–6 weeks, followed by a further injection 7–12 months later. Immunity can be reinforced or "boosted" by subsequent single injections, usually given a number of years later.

has been reduced to 25 if more than one dose is recommended for primary immunization.

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

Individual countries may wish to adopt these Requirements as the basis of their national regulations on tetanus vaccine. If national requirements differ from these requirements, it is recommended that the former should be shown to ensure that the vaccine is at least as safe and as potent as that prepared in accordance with the requirements formulated below. It is desirable that the World Health Organization should be kept informed of any such differences.

PART A. MANUFACTURING REQUIREMENTS

A.1 Definitions

A.1.1 International name and proper name

The international name shall be *Vaccinum tetani adsorbatum*. The proper name shall be the equivalent of the international name in the language of the country of use.

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

A.1.2 Descriptive definition

Vaccinum tetani adsorbatum is a preparation of tetanus toxoid prepared by treating tetanus toxin by chemical means to render it nontoxic without losing its immunogenic potency. The toxoid is adsorbed on to a suitable adjuvant. The preparation shall satisfy the requirements formulated below.

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

A.1.3 International reference materials

The first International Reference Reagent of Tetanus Toxoid for Flocculation Tests was established in 1988 (*6*).

The second International Standard for Tetanus Toxoid, Adsorbed, was established in 1981 (*11*) for determining the potencies of vaccines containing tetanus toxoid. In view of the fact that different results may be obtained when potency tests are carried out in mice instead of guinea-pigs, tests in mice of vaccines containing tetanus toxoid should be performed using reference vaccines calibrated against the International Standard by means of potency tests on guinea-pigs; however, this is not an entirely satisfactory procedure.

The second International Standard for Tetanus Antitoxin was established in 1969 (*12*); it has an *in vivo/in vitro* ratio of antitoxin activity of 1.4 and is made of purified hyperimmune horse serum.

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

A.1.4 Terminology

Seed lot: A quantity of bacterial suspension that is derived from one strain, has been processed as a single lot and has a uniform composition. It is used for preparing the inoculum for the production medium.

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

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

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

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

A.2 General manufacturing requirements

The general requirements for manufacturing establishments contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply to establishments manufacturing tetanus vaccine with the addition of the following:

All manufacturing processes up to and including the completion of detoxification shall be carried out in completely separate areas and by means of separate equipment.

Written descriptions of procedures for the preparation and testing of tetanus vaccine adopted by a manufacturer together with appropriate evidence that each production step has been validated shall be submitted for approval to the national control authority. Proposals for modifications of the manufacturing and/or control methods shall also be submitted for approval to the national control authority before such modifications are implemented.

A.3 Production control

A.3.1 Control of source materials

A.3.1.1 *Strains of Clostridium tetani*

Strains of *C. tetani* used in preparing tetanus toxoid shall be identified by a record of their history and of all tests made periodically to verify strain characters. The strain shall be maintained as a freeze-dried culture.

> A highly toxinogenic strain of *C. tetani* should be used. A strain that has proved satisfactory in many laboratories is the Harvard strain.

A.3.1.2 *Seed lot system*

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

A.3.1.3 *Culture medium for production of toxin*

It is particularly important to ensure that the final product is free from substances likely to cause toxic or allergic reactions in humans.

The method of detecting these substances should be approved by the national control authority.

> If the medium is prepared from a protein digest, e.g., casein hydrolysate or digested muscle, precautions should be taken to ensure that digestion has proceeded sufficiently. Established limits, if any, for mammalian protein and human blood-group substances in the final vaccine should not be exceeded.

A.3.2 Production precautions

The general production precautions, as formulated in the requirements of Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*), shall apply to the manufacture of tetanus vaccine.

> Suitable methods for the production of tetanus vaccine are given in the *Manual for the production and control of vaccines: tetanus toxoid* (*13*).

Personnel employed in production and quality control must be adequately trained and immunized.

A.3.3 Control of single harvests

Consistency of production shall be demonstrated.

> Consistency may be demonstrated by measuring, e.g., the bacterial growth rate, pH and rate of toxin production.

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

A.3.3.1 *Control of bacterial purity*

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

A.3.3.2 *Filtration*

After having been sampled for control of purity, cultures shall be sterilized by means of filtration. A preservative may be added, but phenol shall not be used for this purpose.

Cultures should be filtered as soon as possible after the end of their incubation period. To facilitate filtration, cultures may be centrifuged, provided that suitable precautions are taken to avoid the formation of potentially hazardous aerosols. A filter aid may be added beforehand.

In some countries, no filter capable of shedding fibres may be used.

A.3.3.3 *Determination of antigen concentration*

The supernatant of the culture prior to inactivation shall be tested by a method approved by the national control authority.

It is advisable to determine the antigen content by measuring the toxin content. This is usually done *in vivo*.

Another suitable method for determining the antigen concentration is the flocculation test described in the *Manual for the production and control of vaccines: tetanus toxoid* (*13*); it should be performed both on the supernatant and, for purposes of comparison, a reference material calibrated against the International Reference Reagent of Tetanus Toxoid for Flocculation Tests, or an equivalent reference material approved by the national control authority.

It is preferable for culture filtrates used in preparing purified toxoid to contain not less than 40 Lf/ml.

Antigen content is a good indicator of consistency of production.

A.3.3.4 *Detoxification and purification of toxin*

Purification may either precede or follow detoxification. Purification before detoxification results in a purer product, but particular care must be taken to avoid reversion to toxin, which may also occur when detoxification precedes purification. The method and agent used for detoxification and the method of purification shall be approved by the national control authority.

After detoxification has been completed, the detoxifying agent shall be removed or neutralized by a method approved by the national control authority.

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

reactions in humans. The rate of detoxification may vary and shall be monitored. Harvests shall not be transferred from the detoxification area until the detoxification has been shown to be complete.

A.3.4 Control of bulk purified toxoid

A.3.4.1 *Preparation*

The bulk purified toxoid shall be prepared from either a single harvest or a pool of single harvests, and shall be sterile. Phenol shall not be used as a preservative.

It is advisable to sterilize the bulk purified toxoid by filtration. A preservative approved by the national control authority may be added to the bulk toxoid.

A.3.4.2 *Sterility*

Each bulk toxoid shall be tested for bacterial and mycotic sterility in accordance with the requirements of Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances (*9*)) or by a method approved by the national control authority. If a preservative has been added to the purified bulk, appropriate measures shall be taken to prevent any interference by it in the sterility test.

A.3.4.3 *Specific toxicity*

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

A.3.4.4 *Reversion to toxicity*

Each bulk purified toxoid shall be tested to ensure that reversion to toxin cannot take place on storage. The bulk toxoid shall be diluted in order to obtain the same concentration and chemical environment as those present in the final bulk vaccine, except for the presence of adjuvant.

To determine whether reversion has occurred, diluted toxoids which have been stored at 37 °C for six weeks shall be tested. The test employed shall be approved by the national control authority and shall be sufficiently sensitive to detect very small amounts of toxin. No toxicity shall be detected.

In one country, the test is performed on toxoids that have been stored at 34 °C.

Similar dilutions of toxoid held at 2–8 °C during the same period of time as those held at 34 °C or 37 °C may be tested as controls.

Mice are not as sensitive to tetanus toxin as guinea-pigs but may be used for the test, subject to the approval of the national control authority.

A.3.4.5 *Antigenic purity*

Each bulk purified toxoid shall be tested for antigenic purity by determining the Lf value and the concentration of protein (nondialysable) nitrogen. The Lf value shall be determined by comparison with a reference material calibrated against the International Reference Reagent of Tetanus Toxoid for Flocculation Tests or an equivalent reference preparation approved by the national control authority. The method of testing shall be approved by the national control authority. The bulk purified toxoid shall pass the test if it contains no fewer than 1000 Lf per mg of protein (nondialysable) nitrogen.

Preparation of toxoid containing more than 1500 Lf per mg is both feasible and desirable.

An indication of the antigenic quality of the toxoid may be obtained by measuring the total combining power and expressing it in relation to the number of Lf units. A suitable method for measuring the total combining power is given in the *Manual for the production and control of vaccines: tetanus toxoid* (*13*).

A.3.5 Control of final bulk

A.3.5.1 *Preparation*

The final bulk shall be prepared from bulk purified toxoid. The number of Lf in a single human dose shall be approved by the national control authority but shall not exceed 25 if more than one dose is recommended for primary immunization.

A.3.5.2 *Preservative*

If the vaccine is to be filled in multidose containers, a suitable antimicrobial preservative shall be added. The amount of preservative in the final bulk shall have been shown to have no deleterious effect on the toxoid or on other vaccine components with which the toxoid may be combined, and to cause no unexpected adverse reactions in humans. The preservative and its concentration shall be approved by the national control authority.

Phenol shall not be used as a preservative.

A.3.5.3 *Adjuvants*

The adjuvants used, their purity and their concentration shall be approved by the national control authority.

> Aluminium or calcium compounds are generally used as mineral carriers.

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

> In some countries, these upper limits for the concentration of mineral carriers are considered to be too high and the limits are set at about half those given above.
>
> In some countries, the adsorbent is precipitated in the presence of the toxoid.

The formulation shall be such that the vaccine remains suspended for a reasonable time after shaking.

A.3.5.4 *Sterility*

Each final bulk shall be tested for bacterial and mycotic sterility in accordance with the requirements of Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General

Requirements for the Sterility of Biological Substances, revised 1973) (*9*) or by a method approved by the national control authorities. If a preservative has been added to the final bulk, adequate measures shall be taken to prevent any interference by it in the sterility test.

A.3.5.5 *Specific toxicity*

In some countries, each final bulk is tested for specific toxicity in at least five guinea-pigs, each weighing 250–350 g. Each guinea-pig is given a subcutaneous injection of a quantity equivalent to at least five single human doses, and is then observed daily and examined closely every week for signs of tetanic paralysis. Animals that die are examined by autopsy. The final bulk passes the test if no guinea-pig shows paralysis or any other signs of tetanus within 21 days of injection and if at least 80% of the animals survive the test period. The guinea-pigs must not have been used previously for experimental purposes.

A.3.5.6 *Potency*

The immunizing potency of each final bulk shall be determined by comparison with an appropriate reference material properly calibrated against the International Standard for Tetanus Toxoid, Adsorbed. The test shall involve the inoculation of guinea-pigs or mice with appropriate doses or dilutions of both the final product and the reference material. After immunization, the animals shall be bled or challenged by the subcutaneous route (*10*). If animals are bled, the antitoxin levels of the individual animals may be titrated by means of toxin neutralization tests performed using *in vivo* or *in vitro* serological methods that have been validated on vaccines of the type being tested. Appropriate statistical methods shall be used to calculate the potency of the final bulk. The method adopted and the interpretation of the results shall be approved by the national control authority.

Care should be taken to ensure that diluents are inert and not pyrogenic. Phosphates might interfere with the adsorption of toxoid.

When consistency of production and testing have been established, the numbers of animals injected with each dilution of product may be reduced to levels substantially lower than those originally needed for the three-dilution assays described in the *Manual of details of tests required on final vaccines used in the WHO Expanded Programme on Immunization* (*10*), provided

that the resulting assays are statistically valid. Test methods based on individual quantification of antitoxin allow the use of fewer animals than are needed in challenge tests.

Depending on the purpose, two types of potency assays may be consideree.

Three-dilution assayc m!y be used to test consistency of production and product stacility, and to calibrate reference preparations.

One-dilution assays[1] for evaluating the response based on the same principle as the three-dilution assays may be used at the discretion of the national control authority for the routine testing of vaccine lots of a given formulation as soon as the production process has been established and consistency in production and control has been demonstrated. The test involves the selection of a dose of reference vaccine, expressed as a fraction of 40 IU (i.e., of the minimum potency of a single human dose) that elicits a minimal protective effect in mice or guinea-pigs, and comparing its effect with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the latter is significantly greater than that to the former ($P \leqslant 0.05$), the potency of the test vaccine is satisfactory. One-dilution tests offer advantages only when vaccine potencies are consistently and substantially in excess of 40 IU per single human dose.

The potency of the final bulk shall be approved by the national control authority. The potency of tetanus vaccine used for the immunization of children shall not be less than 40 IU per single human dose. For three-dilution assays, the limits of the 95% confidence intervals of the estimate of potency shall be within 50–200% of the estimated potency unless the lower limit of the 95% confidence interval of the estimated potency is greater than 40 IU per single human dose. When one-dilution tests are performed, the potency of the test vaccine shall be demonstrated to be significantly greater than 40 IU per human dose.

In some countries, potency testing is not carried out on each final bulk but on each final lot.

A.3.5.7 *Amount of residual free detoxifying agent*

The amount of residual free detoxifying agent in each final bulk shall be determined by a method approved by the national control

[1] Information on one-dilution assay methods is given in document BS/89.1618, available on request from Biologicals, World Health Organization, Geneva, Switzerland.

authority and, if formaldehyde has been used, the residual content shall be not more than 0.2 g/l.

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

In some countries, the amount of residual free detoxifying agent is determined in the purified bulk.

If applicable, appropriate tests for the presence of other detoxifying agents (e.g., glutaraldehyde) shall be performed. The tests used and the maximum permissible concentrations of such chemicals shall be approved by the national control authority.

A.3.5.8 *pH*

The pH of the final bulk shall be measured.

The pH should be between 6.0 and 7.0.

A.4 Filling and containers

The requirements applicable to filling and containers given in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply.

Single-dose or multiple-dose containers may be used. Vaccines in multidose containers shall contain a suitable antimicrobial preservative.

A.5 Control of final product

A.5.1 Identity

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

Flocculation in solution, immunoprecipitation of the toxoid in gels or any other specific interaction between the vaccine and tetanus antitoxin may serve as an identity test. Tests on toxoids adsorbed on to aluminium or calcium carriers may be performed after the carrier has been dissolved, or the adsorbed toxoid wholly or partially eluted by sodium citrate at pH 9.

If adequate quantities of toxoid cannot be recovered from the adsorbed vaccine, specific antitoxin may be sought in the sera of animals used in the innocuity test.

A.5.2 Sterility

Final containers shall be tested for bacterial and mycotic sterility by a method approved by the national control authority.

Many countries have regulations governing the sterility testing of the final product. Where these do not exist, the requirements published by WHO shall be met (*9*). If a preservative has been added to the vaccine, appropriate measures shall be taken to prevent any interference by it in the sterility test.

A.5.3 Potency

A potency test shall be carried out as provided in Part A, section A.3.5.6, on each final lot, if such a test has not been performed on the final bulk.

A.5.4 Innocuity

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

A.5.5 Adjuvant content

The adjuvant content of each final lot shall be determined by a method approved by the national control authority (see Part A, section A.3.5.3).

In some countries, this test is used to verify the homogeneity of filling.

A.5.6 Preservative content

The preservative content of each final lot shall be determined (see Part A, section A.3.5.2). The method used shall be approved by the national control authority.

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

A.5.7 pH

The pH of each final lot shall be measured.

The pH should be between 6.0 and 7.0.

A.5.8 Inspection of final containers

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

A.6 Records

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (7) shall apply.

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

A model of a suitable summary protocol to be used for tetanus vaccines is given in Appendix 3.

A.7 Samples

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

A.8 Labelling

The label printed on or affixed to each container and the label on the carton enclosing one or more containers shall show as a minimum:

- the words *Vaccinum tetani adsorbatum* and/or the proper name of the product,
- the name and address of the manufacturer,

- the number of the final lot,
- the recommended storage temperature and the expiry date if kept at that temperature, and
- the recommended single human dose and route of administration.

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

- a statement that the vaccine satisfies the requirements of this document,
- the nature and amount of any preservative present in the vaccine,
- the nature and amount of the adsorbing agent,
- the recommended temperature for storage and transport,
- a warning that the adsorbed vaccine should not be frozen,
- a warning that the adsorbed vaccine should be shaken before use, and
- instructions for the use of the vaccine and information on contraindications and the reactions that may follow vaccination.

A.9 Distribution and transport

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply.

A.10 Stability, storage and expiry date

A.10.1 Stability

Tests shall be conducted to determine the loss of potency to be expected during storage. The stability of the vaccine shall be demonstrated to the satisfaction of the national control authority; final containers from at least three lots derived from different lots of purified bulk toxoid shall be tested on the expiry date to demonstrate stability during storage. The vaccine shall meet the requirements for the final product (see Part A, sections A.5.3, A.5.4, A.5.7 and A.5.8) up to the expiry date, provided that it has been stored at the recommended temperature. When any changes are made in the

production procedure that may affect the stability of the product, the vaccine produced by the new method shall be shown to be stable.

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

A.10.2 Storage conditions

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

Adsorbed vaccines shall not be frozen.

A.10.3 Expiry date

The expiry date shall be approved by the national control authority based on the stability studies referred to in section A.10.1 and shall relate to the date of the last satisfactory potency determination, performed in accordance with Part A, section A.5.3, i.e., the date on which the test animals were immunized with the vaccine.

PART B. NATIONAL CONTROL REQUIREMENTS

B.1 General

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply.

The detailed production and control procedures and any significant changes in them shall be discussed with and approved by the national control authority, which shall obtain the International Standard for Tetanus Toxoid, Adsorbed and establish a national working reference preparation by comparison with it.

B.2 Official release and certification by the national control authority

A vaccine shall be released only if it satisfies Part A of the present Requirements.

A statement signed by the appropriate official of the national control authority shall be provided at the request of the manufacturing establishment and shall certify that the lot of vaccine in question satisfies all national requirements as well as Part A of the present Requirements. The certificate shall state the number under which the lot was released by the national control authority, and the number appearing on the labels of the containers. The official national release document shall be provided to importers of tetanus vaccines.

The purpose of the certificate is to facilitate the exchange of tetanus vaccines between countries. A model of a suitable certificate is given in Appendix 2.

REQUIREMENTS FOR PERTUSSIS VACCINE

GENERAL CONSIDERATIONS

The formulation of the Requirements for [whole-cell] Pertussis Vaccine and the events leading up to their formulation have already been described (*1*). These vaccines have been in use on a wide scale for almost 30 years and, where vaccines of adequate potency have been administered correctly in accordance with correct schedules, the incidence of whooping cough has decreased markedly.

However, two factors have given rise to concern among public health administrators, namely the toxicity of the vaccines and the lack of efficacy of some of them. Whole-cell pertussis vaccines consist of a suspension of killed organisms, and their inoculation has been temporally associated with adverse events. There appears to be no likelihood that vaccines, either whole-cell or acellular, that are effective but cause few adverse reactions can be produced in the immediate future. The aim of these Requirements is both to encourage the production of such vaccines in the longer term and to indicate where further research may be helpful.

Before release, whole-cell pertussis vaccines must be shown to be potent by the mouse protection test. The best evidence that this test is a good indicator of clinical efficacy was provided by the Medical Research Council trial carried out in the United Kingdom from 1951 to 1959, from which it was concluded that vaccines shown to protect mice against intracerebral challenge also protected immunized children against whooping cough when such children were exposed to the disease in the home by infection from a sibling. The establishment of this correlation between test results and efficacy was a marked advance in the development of whole-cell pertussis vaccines against whooping cough, but a number of anomalies still demand an explanation.

A great deal of effort has been put into attempts to obtain greater reproducibility in mouse protection tests. The use of healthy mice selected at random for their place in the test has improved the reproducibility of the results, which are also affected by the particular strain of mice selected, so that great attention should be paid to these parameters of the test.

Although the mouse protection test is still the only recognized test, further research aimed at the establishment of alternative or

supplementary tests should be actively encouraged. Until an alternative test has been shown to be good indicator of efficacy in humans, however, the mouse protection test will remain the only recognized test for the measurement of the potency of whole-cell pertussis vaccines. A description of the test is included in these Requirements.

The presence of agglutinogens 1, 2 and 3 in whole-cell pertussis vaccines is believed to contribute to their protective efficacy, and a test has been introduced in these revised Requirements for the purpose of determining whether such agglutinogens are present before adjuvant is added.

An immunizing dose of pertussis vaccine is the minimum number of killed organisms that have been shown to give an adequate antigenic stimulus and thus provide protection. The number of killed organisms required for this purpose is indicated by the opacity of the bacterial suspension, determined before the bacteria are killed.

Tests for toxicity continue to pose a problem, since a large number of toxins may be produced during the growth of pertussis organisms. Some of these, such as the heat-labile (i.e., dermonecrotic) toxin can be measured quite accurately, whereas others are difficult to quantify. The present Requirements can do no more than specify the mouse weight-gain test adopted by many manufacturers and required by many control authorities as an "in process" control measure. This should not be an impediment to further developments in the measurement of the toxicity of pertussis vaccines.

These revised Requirements propose tests for the heat-labile toxin, the lymphocytosis promoting factor and the endotoxin for use in monitoring detoxification processes, as well as for validating methods used for detoxification and establishing consistency of production.

Since the requirement previously included for the abnormal toxicity test (intraperitoneal injection of mice with a single human dose) gives rise to excessive vaccine-related symptoms of toxicity, these revised Requirements specify the intraperitoneal injection of only half a single human dose.

Each of the following sections constitutes a recommendation. Those parts of each section printed in large type have been written in the form of requirements so that, if a health administration so desires, they may be adopted as they stand as definitive national

requirements. Those parts of each section printed in small type are comments and/or recommendations for guidance.

Individual countries may wish to adopt these Requirements as the basis of their national regulations on pertussis vaccines. If national requirements differ from these requirements, it is recommended that the former should be shown to ensure that the vaccine is at least as safe and as potent as that prepared in accordance with the requirements formulated below. It is desirable that the World Health Organization should be kept informed of any such differences.

PART A. MANUFACTURING REQUIREMENTS

A.1 Definitions

A.1.1 International name and proper name

The international name shall be *Vaccinum pertussis*. The proper name shall be the equivalent of the international name in the language of the country of use.

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

A.1.2 Descriptive definition

Vaccinum pertussis is a saline suspension of the whole cells of one or more strains of killed *Bordetella pertussis* which have been appropriately treated to minimize toxicity and retain potency. The preparation shall satisfy all the requirements formulated below.

A.1.3 International reference materials

The second International Standard for Pertussis Vaccine was established in 1980 (*14*). It is intended for the calibration of national standards used for determining the potencies of pertussis vaccines. It is in the custody of the International Laboratory for Biological Standards, State Serum Institute, Copenhagen.

The fifth International Reference Preparation of Opacity (*15*) consists of plastic rods simulating the optical properties of a bacterial suspension and defined as having an opacity of 10 International Units of opacity. Countries are invited to use the

International Reference Preparation of Opacity so that the opacities of suspensions of pertussis bacteria can be expressed in International Units of opacity. A bacterial suspension having the same opacity as the International Reference Preparation of Opacity has a bacterial concentration providing 10 IU of opacity. The relationship between such units and actual numbers of bacterial cells may vary from vaccine to vaccine.

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

International reference materials are distributed free of charge, on request, to national control laboratories.

A.1.4 Terminology

Seed lot: A quantity of bacterial suspension that is derived from one strain, has been processed as a single lot and has a uniform composition. It is used for preparing the inoculum for the production medium.

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

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

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

A.2 General manufacturing requirements

The general requirements for manufacturing establishments contained in the Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply to establishments manufacturing pertussis vaccine, with the addition of the following:

Written descriptions of procedures for the preparation and testing of pertussis vaccine adopted by a manufacturer together with appropriate evidence that each production step has been validated shall be submitted for approval to the national control authority. Proposals for modifications of the manufacturing and/or control

method shall also be submitted for approval to the national control authority before such modifications are implemented.

A.3 Production control

A.3.1 Control of source materials

A.3.1.1 *Strains of Bordetella pertussis*

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

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

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

A.3.1.2 *Seed lot system*

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

A.3.1.3 *Culture medium for production of bacteria*

The medium shall enable *B. pertussis* to grow and to retain agglutinogens and potency. Human blood or blood products shall not be used in culture media for propagating bacteria, either for seed or for vaccine. When animal blood or blood products are used, they shall be removed by washing the harvested bacteria, and the final product shall be demonstrated to be free of contaminating antigens and allergenic substances.

The absence of sensitizing animal proteins in final vaccines may be demonstrated by passive cutaneous anaphylaxis or other suitably sensitive and specific procedures. In some countries, the use in the medium of blood from any source is not permitted.

A.3.2 Production precautions

The general production precautions, as formulated in the requirements of Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories (*7*) shall apply to the manufacture of pertussis vaccine.

A.3.3 Control of single harvests

Consistency of production shall be demonstrated.

> Consistency may be evaluated by measuring the bacterial growth rate and testing for the presence of agglutinogens in the cultured organisms.

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

A.3.3.1 *Control of bacterial purity*

Samples of single harvests taken before killing shall be tested for purity by microscopic examination of stained smears or by inoculation into appropriate culture media. Single harvests shall not be used for the final bulk if contamination has occurred at any stage in their production.

A.3.3.2 *Control of opacity*

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

A.3.3.3 *Killing and detoxification*

After samples of single harvests have been taken for purposes of purity control and opacity measurement, the bacteria shall be killed and detoxified by a method approved by the national control authority. If chemicals are used for this purpose, they shall be approved by the national control authority. In order to ensure that

the organisms have been killed, a sample shall be tested in an appropriate culture medium.

B. pertussis can be killed by a number of methods whose effectiveness depends on the concentration of the chemicals used and the temperature, time and pH at which killing is carried out. The aim in killing and detoxification is to achieve complete killing and an appropriate level of detoxification without adversely affecting the potency or the physical characteristics of the vaccine. The methods used should be validated to the satisfaction of, and approved by, the national control authority.

After killing and detoxification, the opacity of the suspension will be different from what it was originally. Each single harvest should, however, still be regarded as containing the same number of bacteria.

No biologically active heat-labile toxin (dermonecrotic toxin) should be detectable in a vaccine. The method of manufacture should have been shown to ensure that active dermonecrotic toxin is not present in the final product. The method of detoxification used should minimize the bioactivity of lymphocytosis promoting factor while retaining immunogenicity. Since endotoxin (lipopolysaccharide, LPS) is an intrinsic part of the cell envelope of *B. pertussis*, it is best controlled by reducing the number of bacteria needed to achieve an acceptable level of potency (see also section A.3.4.7). In the present state of knowledge, it is not possible to recommend limits for levels of lymphocytosis promoting factor, endotoxin, tracheal cytotoxin and adenylate cyclase in whole-cell pertussis vaccines.

A.3.4 Control of final bulk

A.3.4.1 *Preparation*

The final bulk shall be prepared by pooling a number of single harvests. Where vaccine is prepared from two or more strains, consecutive batches of the final bulk shall be consistent with respect to the proportions of each strain present, as measured in opacity units. The concentration of bacteria in the final bulk, when prepared as a formulation for a single human dose of 1 ml, shall correspond to an opacity (before killing) of not more than 20 IU. The concentration of bacteria in the final bulk, when prepared as a formulation for a single human dose of 0.5 ml, shall correspond to an opacity (before killing) of not more than 40 IU. The number of IU of opacity in the final bulk shall be calculated from that found in the tests (see Part A, section A.3.3.2) performed on the single harvests.

It is advisable to use as few IU of opacity as possible while still satisfying the potency requirements prescribed in section A.3.4.7. Potent vaccines which contain in a single human dose the equivalent of a 1-ml suspension having 5–10 IU of opacity can be produced consistently and manufacturers should be encouraged to produce potent vaccines of even lower opacity. In some countries, vaccines containing an aluminium carrier must not contain in a single human dose more than the equivalent of 1 ml of a suspension having 16 IU of opacity.

A.3.4.2 *Agglutinogens*

Each bulk shall be examined for the presence of agglutinogens 1, 2 and 3 before adjuvant is added.

A.3.4.3 *Preservative*

If the vaccine is to be dispensed into multidose containers, a suitable antimicrobial preservative shall be added. The amount of preservative in the final bulk shall have been shown not to have any deleterious effect on the pertussis immunogen or on other vaccine components with which pertussis vaccine may be combined, and not to cause any unexpected adverse reactions in humans. The preservative and its concentration shall be approved by the national control authority.

Phenol shall not be used as a preservative.

A.3.4.4 *Adjuvants*

Adjuvants may be added to the vaccine; their nature, purity and concentration shall be approved by the national control authority.

Aluminium or calcium compounds may be used as mineral carriers.

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

In some countries, an upper limit of 1.25 mg of aluminium is considered to be excessive for products containing a pertussis component and such vaccines contain only 0.1–0.3 mg of aluminium per single human dose.

The formulation shall be such that the vaccine remains suspended for a reasonable time after shaking.

A.3.4.5 *Sterility*

Each final bulk shall be tested for bacterial and mycotic sterility in accordance with the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (*9*) or by a method approved by the national control authority. If a preservative has been added to the vaccine, appropriate measures shall be taken to prevent any interference by it in the sterility test.

A.3.4.6 *Specific toxicity*

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

Toxicity tests in animals are required by many national control authorities, but in most tests the results obtained depend to some extent on factors independent of the vaccine, such as the strain of animal used and the conditions under which the animals are kept.

Little information is available on the relationship between the toxicity of vaccines in animals and the occurrence of untoward reactions following vaccination in humans.

Mouse weight-gain test. One of the tests most widely used is the mouse weight-gain test; it has been of some value in ensuring the production of vaccines which, in general, are satisfactory in that they cause minimal untoward reactions in humans. The test may be performed as follows. No fewer than 10 healthy mice each weighing 14–16 g are used for each sample and for the saline control. They should have access to food and water for at least 2 h before injection and continuously after injection for the duration of the test. The total weight of the group of mice is determined immediately before injection. The mice used for testing the vaccine(s) and the control group of mice should be of the same sex. If both sexes are used, they should be equally distributed in all groups. Each mouse is given an intraperitoneal injection of 0.5 ml of a suspension containing a volume of the final bulk under test equivalent to not less than half the volume recommended as a single human dose. A similar control group of mice is inoculated with 0.5 ml of physiological saline, preferably containing the same amount of preservative as the inoculum injected into the test mice. The total weight of each group of mice is determined 72 h and seven days after injection. The final bulk is considered to be satisfactory if: (*a*) at the end of 72 h the total weight of the group is not less than it was before the injection; (*b*) at the end of seven days, the average weight gain per mouse is not less than 60% of that of the control group

of mice; and (*c*) not more than 5% of the total number of injected mice die.

Other tests. Cell harvests of *B. pertussis* to be used in the manufacture of pertussis vaccine contain a number of biologically active molecules which may contribute to the toxicity of final product. Assays for some of these substances can be used to monitor and validate the methods used for detoxification and may also be useful in assessing final products. In the process of validating the manufacturing procedures, manufacturers are encouraged to measure at least one of the following:

(1) *Heat-labile toxin* (dermonecrotic toxin). Subcutaneous injection of test materials into the nuchal area of suckling mice is the most sensitive and useful method of detecting heat-labile toxin. The suckling mouse test should be used to validate the manufacturing process but need not be performed on the final bulk or final container materials. Pertussis vaccine should not contain biologically active heat-labile toxin.

(2) *Lymphocytosis promoting factor.* Many different assays can be used to measure the diverse activities of lymphocytosis promoting factor but many suffer from technical problems which make their use as routine control procedures difficult. The induction of lymphocytosis in mice is not a sensitive method but is an adequate means of monitoring the level of active factor in bulk vaccine. Tests for histamine sensitizing activity in mice may also be used.

(3) *Endotoxin.* The endotoxin content of vaccines can be determined by the limulus amoebocyte lysate assay, the rabbit pyrogen test and the silver staining of electrophoretic gels. However, there is no agreement as to what constitutes an acceptable level of endotoxin in whole-cell pertussis vaccines.

A.3.4.7 *Potency*

The potency of each final bulk (or of each final lot) shall be determined by comparison with that of a reference vaccine calibrated against the International Standard for Pertussis Vaccine or an equivalent standard vaccine approved by the national control authority. The assay shall be performed by the intracerebral mouse protection test. The assay method and the method of calculating the results shall be approved by the national control authority. The potency of the final bulk shall be not less than 4.0 IU in the volume recommended as a single human dose.

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

(*a*) *Mice.* Healthy mice, preferably from a strain and colony capable of giving an adequate immune response, are used. They should preferably be of the same sex but, if this is not possible,

both sexes should be distributed equally throughout the test and the sexes segregated. Each mouse should weigh at least 10 g but not more than 18 g and, in a single test, the mice should not differ in weight by more than 4 g.

The mice are randomly allocated to the different groups, and the shelf position of the cages, the order of immunization, and the order of challenge are also randomized. Groups of at least 16 mice should be used for each dilution of the standard vaccine and of the vaccines under test, and at least 16 mice should be used for each dilution of the culture in the determination of the challenge dose.

(*b*) *Immunization of mice.* At least three dilutions of the reference vaccine and of each lot of vaccine should be tested. Serial dilutions, not greater than five-fold, of the vaccine to be tested and of the standard vaccine should be made in a suitable diluent. The median effective dose (ED_{50}) for each preparation should be bracketed by the dilutions used. Each mouse in each immunization group should be injected intraperitoneally with 0.5 ml of the appropriate dilution.

The interval between immunization and challenge should be 14–17 days. At least 94% of the mice immunized by each dilution of both the reference vaccine and the vaccines under test should survive until challenged, and each mouse challenged should appear healthy prior to challenge.

(*c*) *The challenge.* The strain used for challenge (generally *B. pertussis* 18323) should be approved by the national control authority. To ensure constancy of virulence from test to test, a large working challenge lot prepared from the master culture is dispensed into ampoules and freeze-dried or stored in liquid nitrogen.

The bacterial suspension used for challenge is prepared from a 20–24-h culture grown on Bordet-Gengou medium, or other suitable medium that has been seeded from a rapidly growing culture not more that 30 h old. Alternatively, aliquots of the challenge suspension may be frozen and kept in liquid nitrogen; after thawing and dilution, they can be used directly as the challenge culture. The suspension is diluted with a diluent in which the organisms will remain viable, e.g., an aqueous solution containing 10 g/l casein peptone and 6 g/l sodium chloride adjusted to a pH of 7.1 $\pm$ 0.1. The suspension, free from particles of agar or clumps of bacteria, is adjusted in such a way that each challenge dose of not more than 0.03 ml contains 100–1000 times the median lethal dose (LD_{50}).

Mice immunized with the reference vaccine and the vaccines under test are challenged at random under mild narcosis by intracerebral injection of the challenge dose. To obtain an estimate of the LD_{50}, dilutions of the challenge dose are then injected into control mice by the intracerebral route and an appropriate dilution of the challenge dose is cultured on Bordet-

Gengou medium to determine the number of colony-forming units contained therein.

(*d*) *Recording of results.* The mice are observed for 14 days. Mice dying within 72 hours should be excluded from the test. To determine the ED_{50} of the vaccines, records should be kept of the number of mice that die after 72 hours.

(*e*) *Calculation of results.* The ED_{50} values for each preparation are determined by a statistical method that includes the transformation of the mouse survival data into a form capable of consistently producing a linear regression. Probits, logits and angle transformation have been shown to be suitable. Similar methods should be used to determine the LD_{50} of the challenge suspension.

(*f*) *Validity of the test.* The test is valid if the ED_{50} of each vaccine is intermediate between the largest and the smallest immunizing doses, and the regressions do not show significant deviations from linearity and parallelism ($P \leqslant 0.05$). The challenge dose should contain 100–1000 LD_{50} and the LD_{50} should contain no more than 300 colony-forming units.

(*g*) *Estimate of potency.* The ED_{50} of the vaccine under test and the standard vaccine are calculated by a method that provides an estimate of the limits of the 95% confidence intervals (*10*). The potency is estimated in terms of International Units in the volume recommended for a single human dose, unless the national control authority decides otherwise. The vaccine passes the requirements for potency if the result of a statistically valid test shows that the estimated potency of the vaccine is not less than 4.0 IU in the volume recommended for a single human dose and if the lower fiducial limit ($P = 0.95$) of the estimated potency is not less than 2.0 IU. Additional tests may be carried out, but in this case the results of all valid tests must be combined in the geometric mean estimate and its lower fiducial limit. In some countries, an upper limit of potency is also specified.

A.3.4.8 *pH*

The pH of the final bulk shall be measured.

A.4 Filling and containers

The requirements applicable to filling and containers given in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply.

Single-dose and multiple-dose containers may be used. Vaccine in multidose containers shall contain a suitable antimicrobial preservative.

A.5 Control of final product

A.5.1 Identity

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

> Agglutination of the organisms with specific antipertussis serum may serve as an identity test. Vaccines may also be inoculated into animals in order to show that pertussis agglutinins are produced in their serum.

A.5.2 Sterility

Final containers shall be tested for sterility by a method approved by the national control authority.

Many countries have regulations governing the sterility testing of the final product. Where these do not exist, the requirements published by WHO shall be met (*9*). If a preservative has been added to the vaccine, appropriate measures shall be taken to prevent any interference by it in the sterility test.

A.5.3 Potency

A potency test shall be carried out as provided in Part A, section A.3.4.7, on each final lot, if such a test has not been made on the final bulk.

A.5.4 Innocuity

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

A.5.5 Adjuvant content

If an adjuvant has been added to the final bulk, the adjuvant content of the final lot shall be determined by a method approved by the national control authority (see Part A, section A.3.4.4).

In some countries, this test is applied to verify the homogeneity of filling.

A.5.6 Preservative content

If preservative has been added to the final bulk, the preservative content of the final lot shall be determined (see Part A, section A.3.4.3). The test method shall be approved by the national control authority.

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

A.5.7 pH

The pH of each final lot shall be measured.

The permitted range of pH values shall be approved by the national control authority.

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

A.5.8 Inspection of final containers

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

A.6 Records

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply, with the addition of the following:

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

A model of a suitable summary protocol to be used for pertussis vaccines is given in Appendix 4.

A.7 Samples

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

A.8 Labelling

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

- the words *Vaccinum pertussis* and/or the proper name of the product,
- the word "adsorbed", if applicable,
- the name and address of the manufacturer,
- the recommended storage temperature and the expiry date if kept at that temperature, and
- the recommended single human dose and route of administration.

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

- a statement that the vaccine satisfies the requirements of this document,
- the nature and amount of any preservative present in the vaccine (if there is no preservative in single-dose containers, this should be stated),
- the nature and amount of the adsorbing agent, if applicable,
- the nature and amount of any substances added to the vaccine,
- the recommended conditions for storage and transport,
- a warning that the vaccine should not be frozen,
- a warning that the vaccine should be shaken before use, and
- instructions for the use of the vaccine and information on contraindications and the reactions that may follow vaccination.

A.9 Distribution and transport

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Require-

ments for Manufacturing Establishments and Control Laboratories) (7) shall apply.

A.10 Stability, storage and expiry date

A.10.1 Stability

Tests shall be conducted to determine the loss of potency to be expected during storage. The stability of the vaccine shall be demonstrated to the satisfaction of the national control authority; final containers from at least three batches of vaccine derived from different bulks shall be tested on the expiry date to demonstrate stability during storage. The vaccine shall meet the requirements for final vaccine (see Part A, sections A.3.4.7, A.5.4, A.5.7 and A.5.8) up to the expiry date, provided that it has been stored at the recommended temperature. When any changes are made in the production procedure which may affect the stability of the product, the vaccine produced by the new method shall be shown to be stable.

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

A.10.2 Storage conditions

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

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

The vaccine shall not be frozen.

A.10.3 Expiry date

The expiry date shall be fixed with the approval of the national control authority based on the stability studies referred to in section A.10.1 and shall not be more than 2½ years after the date of the last satisfactory potency test, i.e., the date on which the animals were immunized with the vaccine.

PART B. NATIONAL CONTROL REQUIREMENTS

B.1 General

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (*7*) shall apply.

In view of the lack of information on the relationship between the toxicity of vaccines in animals and the production of untoward reactions in humans after vaccination, and the sometimes wide limits of the 95% confidence interval of the potency data (Part A, section A.3.4.7), the degree of consistency in producing satisfactory final bulk vaccines is an important factor in ensuring the safety and efficacy of a particular manufacturer's product. Definite requirements in this respect cannot be formulated, but the national control authorities should satisfy themselves, on the basis of the method of manufacture and the results of tests on a series of consecutive vaccines, that the manufacturer is able to produce, with satisfactory consistency, a product of the required quality.

The detailed production and control procedures and any significant changes in them shall be discussed with and approved by the national control authority, which shall obtain the International Standard for Pertussis Vaccine and establish a national working reference preparation by comparison with it.

B.2 Official release and certification by the national control authority

A vaccine shall be released only if it satisfies Part A of the present Requirements.

A statement signed by the appropriate official of the national control authority shall be provided at the request of the manufacturing establishment and shall certify that the lot of vaccine in question satisfies all national requirements as well as Part A of the present Requirements. The certificate shall state the number under which the lot was released by the national control authority, and the number appearing on the labels of the containers. The official national release document shall be provided to importers of pertussis vaccines.

The purpose of the certificate is to facilitate exchange of pertussis vaccines between countries. A model of a suitable certificate is given in Appendix 2.

REQUIREMENTS FOR COMBINED VACCINES (ADSORBED)

GENERAL CONSIDERATIONS

In order to satisfy the need for combined vaccines, national control authorities have combined the appropriate tests for the individual components of diphtheria and tetanus (DT) and diphtheria, tetanus and pertussis (DTP) vaccines, but have not adopted a uniform approach in so doing.

The requirements for a combined vaccine must include the tests applicable to the various components incorporated into the final product, but further tests are also required after blending. The need for tests at each stage in the production is important because of possible interaction between antigens, as well as the effects that both adjuvants and preservatives may have on the potency and stability of the final product.

The tests carried out on the combined vaccines are on the whole the same as those on the individual components, so that, in requirements, a cross-reference to the tests specified for these components, including the "one-dilution" tests, is all that is necessary in many cases. However, certain tests—such as the tetanus potency assay of DTP vaccine—need special consideration when mice are used in the assay.

No international reference materials specific for the combined vaccines exist, and the potency of each component is expressed in International Units by comparison with reference materials calibrated against the reference materials for the individual components. This is not an ideal situation, because dose–response relationships in animals may differ when the components are combined. Nevertheless, meaningful potency data can be obtained.

Filling, sampling, labelling, transportation, distribution and storage have not been dealt with here since they have been adequately covered for the individual (diphtheria, tetanus, pertussis) vaccines. However, it should be noted that, as with single adsorbed vaccines, combined DT and DTP vaccines must not be frozen and that the expiry date of combined vaccines is determined by the component with the shortest shelf-life.

A number of manufacturers and control authorities have experienced difficulties in reporting the results of tests of combined

vaccines, and a composite protocol for this purpose has therefore been included for DTP (Appendix 5).

These Requirements cover only the two combined vaccines DT and DTP, since these combinations are the most widely used. Reference is also made to tetanus and diphtheria vaccine for adults (Td) for which the potency requirements for the diphtheria component are reduced. No attempt has been made to include other combinations, including those with *Haemophilus influenzae* and *Neisseria meningitidis* polysaccharides and poliomyelitis vaccines. Requirements for these vaccines should therefore include the relevant tests for the individual components, and special attention should be given to possible interactions between the components.

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

Individual countries may wish to adopt these Requirements as the basis of their national regulations on combined vaccines. It national requirements differ from these requirements, it is recommended that the former should be shown to ensure that the vaccine is at least as safe and as potent as that prepared in accordance with the requirements formulated below. It is desirable that the World Health Organization should be kept informed of any such differences.

PART A. MANUFACTURING REQUIREMENTS

A.1 Tests for DT and DTP vaccines

A.1.1 Final bulk

The following tests on the final bulk are common to diphtheria, tetanus and pertussis vaccines (DTP) and to diphtheria and tetanus vaccines (DT): the test for adjuvant content, the sterility test and the test for free detoxifying agent.

A.1.2 Final lot

The following tests on the final lot are common to both DTP and DT: the sterility test, the test for adjuvant content, the test for preservative content and the inspection of the final containers. The pH of DT and DTP vaccines shall be 6.0–7.0.

A.2 Special tests for DTP vaccine

A.2.1 Final bulk

The following tests shall be carried out on the final bulk.

A.2.1.1 *Potency test*

For the diphtheria component, the requirements for diphtheria vaccine (section A.3.5.6, p. 101) shall apply.

When the test for the potency of the tetanus component is performed in guinea-pigs, the requirements for tetanus vaccine (section A.3.5.6, p. 119) shall apply. When mice are used, the potency of the tetanus component shall be 60 IU per single human dose. The 95% confidence interval of the tests shall be within 50–200%. If the interval is greater than 50–200%, the lower 95% fiducial limit of the estimate of potency must be greater than 60 IU per dose. In one-dilution tests in mice, the dose of reference vaccine injected shall be expressed as a fraction of 60 IU.

For the pertussis component, the requirements for pertussis vaccine (section A.3.4.7, p. 136) shall apply.

A.2.1.2 *Specific toxicity test*

For the specific toxicity test for the diphtheria component, the requirements for diphtheria vaccine (section A.3.5.5, p. 101) apply.

For the specific toxicity test for the tetanus component, the requirements for tetanus vaccine (section A.3.5.5, p. 119) apply.

The same animals are used for these two tests and are observed for six weeks in order to cover the observation period specified for diphtheria vaccine (section A.3.5.5).

For the mouse weight-gain test for pertussis toxicity, the requirements for pertussis vaccine (section A.3.4.6, p. 135) apply.

A.2.2 Final lot

The following tests shall be carried out on the final lot.

A.2.2.1 *Identity test*

Identity tests on the components of a triple vaccine shall be carried out after they have been eluted from the mineral carrier with sodium citrate and the pertussis organisms and residual carrier sedimented by centrifugation. The tests specified in the requirements for diphtheria vaccine (section A.5.1, p. 104), tetanus vaccine (section A.5.1, p. 121) and pertussis vaccine (section A.5.1, p. 139) shall apply.

A.2.2.2 *Potency test*

If a potency test has not been performed on the triple vaccine in the final bulk, the tests specified in section A.2.1.1 (p. 146) shall apply.

A.2.2.3 *Innocuity test*

The innocuity test shall be performed in accordance with the requirements for pertussis vaccine (section A.5.4, p. 139).

A.3 Special tests for DT vaccine

A.3.1 Final bulk

The following tests shall be carried out on the final bulk.

A.3.1.1 *Potency test*

The test for the potency of the diphtheria component shall be that specified for diphtheria vaccine (section A.3.5.6, p. 101).

> In some countries, tetanus and diphtheria vaccine for use in adults (Td) is released with a diphtheria potency of less than 30 IU per dose.

The test for the potency of the tetanus component of DT and Td vaccines shall be that specified for tetanus vaccine (section A.3.5.6, p. 119).

A.3.1.2 *Specific toxicity test*

The tests for the specific toxicity of the diphtheria and tetanus components are as specified in section A.3.5.5, pp. 101 and 119, with an observation period of six weeks in order to cover the period specified for diphtheria vaccine.

A.3.2 Final lot

The following tests shall be carried out on the final lot.

A.3.2.1 *Identity test*

Identity tests on the components of DT vaccine shall be carried out after they have been eluted from the mineral carrier with sodium citrate. The tests specified for diphtheria vaccine (section A.5.1, p. 104) and tetanus vaccine (section A.5.1, p. 121) shall apply.

A.3.2.2 *Potency test*

If a potency test has not been performed on the final bulk, the tests specified for the individual vaccines (section A.3.5.6, pp. 101 and 119) shall apply.

A.3.2.3 *Innocuity test*

The innocuity test shall be performed in accordance with the requirements for diphtheria and tetanus vaccines (section A.5.4, pp. 105 and 122).

PART B. NATIONAL CONTROL REQUIREMENTS

In addition to its responsibilities in respect of each individual vaccine, including release and certification, the national control authority shall approve:

- the formulation of the combined vaccine in order to ensure that the components are present at concentrations appropriate to its use,
- the formulation including the preservative and adjuvant in order to ensure that the stability of the vaccine is such that it remains effective up to the expiry date, provided that it has been stored at the recommended temperature, and

- the protocols for reporting the results of tests on the combined vaccine (a suggested protocol for this purpose is given in Appendix 5).

AUTHORS

The first drafts of the revised Requirements for Diphtheria, Tetanus, Pertussis and Combined Vaccines were prepared in 1988 by Dr P. Knight (Wellcome Biotech, Beckenham, England) and Dr C. Manclark (Laboratory of Pertussis, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA).

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ACKNOWLEDGEMENTS

Acknowledgements are due to the following experts for their comments and advice on the second draft of these Requirements: Dr R.H. Ali, President, Egyptian Organization for Biological Products and Vaccines, Agouza, Cairo, Egypt; Dr J. Cameron, North York, Ontario, Canada; Dr R. Donikian, Mérieux Institute, Marcy l'Etoile, France; Dr V. Garic, Head, Control Department, Institute of Immunology and Virology, Torlak, Belgrade, Yugoslavia; Dr E.H. Ghaly, Director General of Production, Egyptian Organization for Biological Products and Vaccines, Agouza, Cairo, Egypt; Dr D.K. Gill, Director, Regulatory Affairs, International, Connaught Laboratories Limited, Willowdale, Ontario, Canada; Dr E. Griffiths, Department of Bacteriology, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr C. Guthrie, Operations Director, Commonwealth Serum Laboratories, Parkville, Victoria, Australia; Professor D.K. Hazra, Head, Nuclear Medicine and Radio-immunoassay Unit, Post Graduate Department of Medicine, S.N. Medical College, Agra, India; Dr P. Knight, Wellcome Biotech, Beckenham, Kent, England; Dr Ko Ko Gyi, Director, Development Centre for Pharmaceutical Technology, Rangoon, Myanmar; Dr M. Krasselt, National Institute for Public Health and Environmental Protection, Bilthoven, Netherlands; Dr J. G. Kreeftenberg, Head, Laboratory for Control of Bacterial Vaccines, National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands; Dr D. Labert, Pasteur Vaccines, Val de Reuil, France; Dr S. Langlois, Connaught Laboratories Limited, Willowdale, Ontario, Canada; Mr W. Latham, Massachusetts Public Health Biological Laboratories, Boston, MA, USA; Dr Lehmann, Behringwerke, Marburg, Federal Republic of Germany; Mr J. Lyng, Department of Biological Standardization, State Serum Institute, Copenhagen, Denmark; Mr E. McCarthy, Managing Director, Wyeth-Ayerst Laboratories, Marietta, PA, USA; Dr K. McClintock, Director, Regulatory and Technical Affairs, Lederle Laboratories, Pearl River, NY, USA; Dr D. McKay, National Biological Standards Laboratory, Canberra, Australia; Dr T. Matuhasi, Consulting Research Scientist, Okinaka Memorial Institute for Medical Research, Tokyo, Japan; Dr L. Milcke, Behringwerke, Marburg, Federal Republic of Germany; Dr J. Milstien, Scientist, Biologicals, World Health Organization, Geneva, Switzerland; Dr G. Nyerges, Department of Control of Vaccines and Sera, National Institute of Hygiene, Budapest, Hungary; Dr M. Quevillon, Armand Frappier Institute, Quebec, Canada; Dr K. Readhead, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr P. Rousseau, Armand Frappier Institute, Quebec, Canada; Dr S.N. Saxena, Director, Central Research Institute, Kasauli, India; Dr E.B. Seligmann Jr, Consultant, Biologics Manufacturing, Frederick, MD, USA; Dr D.W. Stainer, Conpharma Vaccines Ltd., North York, Ontario, Canada; Dr G.R.E. Swaniker, Faculty of Medicine, The University of Papua New Guinea, Boroko, Papua New Guinea; Dr V. Takla-Rizk, Egyptian Organization for Biological Products and Vaccines, Agouza, Cairo, Egypt; Dr S.J. Varallyay, Swiss Vaccine and Serum Institute, Bern, Switzerland; Dr J. Vose, Assistant Vice-President, Regulatory and Technical Affairs, Connaught Research Institute, Willowdale, Ontario, Canada; Dr J.J. Walsh, Quality Assurance and Animal Services Director, Commonwealth Serum Laboratories, Parkville, Victoria, Australia; Dr J.C.W. Weber, Director, Regulatory Affairs, Connaught Laboratories Limited, Willowdale, Ontario, Canada; Dr Yu Yong Pin, National Institute for Control of Pharmaceutical and Biological Products, Temple of Heaven, Beijing, China.

REFERENCES

1. WHO Technical Report Series, No. 274, 1964.
2. WHO Technical Report Series, No. 293, 1964.
3. WHO Technical Report Series, No. 638, 1979.
4. *Weekly epidemiological record*, **63**: 317–319 (1988).
5. WHO Technical Report Series, No. 760, 1987.
6. WHO Technical Report Series, No. 786, 1989.
7. WHO Technical Report Series, No. 323, 1966.
8. *Manual for the production and control of vaccines: diphtheria toxoid.* Unpublished WHO document BLG/UNDP/77.1 Rev. 1.[1]
9. WHO Technical Report Series, No. 530, 1973.
10. *Manual of details of tests required on final vaccines used in the WHO Expanded Programme on Immunization.* Unpublished WHO document BLG/UNDP/82.1 Rev. 1.[1]
11. WHO Technical Report Series, No. 673, 1982.
12. WHO Technical Report Series, No. 444, 1970.
13. *Manual for the production and control of vaccines: tetanus toxoid.* Unpublished WHO document BLG/UNDP/77.2 Rev 1.[1]
14. WHO Technical Report Series, No. 658, 1981.
15. WHO Technical Report Series, No. 594, 1976.

[1] Available to readers on request from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

Appendix 1

SUMMARY PROTOCOL FOR DIPHTHERIA VACCINE (ADSORBED) PRODUCTION AND TESTING

Summary information on final lot

Name and address of manufacturer .....
.....
.....

Lot No. .....

Date of filling .....

Volume of each recommended single human dose .....

No. of doses per final container .....

No. of final containers .....

Expiry date .....

Detailed information on manufacture and control

Strain

Identity of *C. diphtheriae* strain used for vaccine production .....

Reference No. of seed lot .....

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

Single harvests used for preparing the bulk purified toxoid

List the single harvests and indicate the medium, dates of inoculation, temperature of incubation, dates of harvests, volumes, results of tests for bacterial purity, method of inactivation and yields.

Bulk purified toxoid

Reference No. .....

Volume and Lf/ml .....

Date and result of test for antigenic purity (Lf/mg of protein nitrogen) .....

Test of irreversibility

Lf/ml of test toxoid solution .....

Temperature of incubation of toxoid .....

Dates of beginning and end of incubation .....

If a test on guinea-pigs was used, indicate:

No. of guinea-pigs injected, route and date of injection .....

Date of end of observation .....

Result of test .....

If cell culture was used, provide suitable information on the test system and give results

Specific toxicity test

If a test on guinea-pigs was used, indicate:

No. of guinea-pigs injected and date of injection .....

No. of Lf per guinea-pig and route of injection .....

Date of end of observation .....

Result of test .....

If cell culture was used, provide suitable information on the test system and give results

Final bulk

Identification .....

Volume .....

Lf/ml .....

Sterility test

Date and result of test .....

Specific toxicity test (optional)

No. of guinea-pigs injected and date of injection .....

Volume and route of injection .....

Date of end of observation .....

Result of test .....

Potency test[1]

(1) Based on lethal or paralytic challenge

(i) Three-dilution assays

(*a*) Lethal challenge

Weight of guinea-pigs .....

Date of immunization and volume of dilutions administered .....

Date of challenge .....

Challenge dose .....

Date of end of observation .....

Results .....

	Dilution	*No. of survivors/No. of animals injected*	*Median effective dose (ED_{50})*
Reference vaccine (... IU/ml)	.....	.....	
	.....	.....	..... ml
	.....	.....	
Test vaccine	.....	.....	
	.....	.....	..... ml
	.....	.....	

Potency of test toxoid is ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

(*b*) Multiple intradermal challenge

Report all relevant information on animals and date of immunization, challenge and end of observation.

Results

	Dilution	*Mean score*
Reference vaccine (... IU/ml)	.....	.....
	.....	.....
	.....	.....
Test vaccine	.....	.....
	.....	.....
	.....	.....

Potency of test toxoid is ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

(ii) One-dilution challenge test

Date of performance of last satisfactory three-dilution test .....

[1] Only one of the potency tests listed need be performed.

Nature and reference No. of product tested (specify also whether it was a final bulk or a final product) .....

Provide relevant information validating the one-dilution assay system.

Identity and titre (IU/ml) of reference vaccine .....

Weight of guinea-pigs .....

Date of immunization .....

Date of challenge .....

Challenge dose .....

Date of end of observation .....

Results

	Reference vaccine	*Test vaccine*
Dilution used for immunization	.....	.....
No. of survivors/No. injected	.....	.....

P value indicating the probability that the test vaccine contains more than 30 IU per single human dose .....

(2) Test based on measuring antitoxin induction as an alternative to lethal or intradermal challenge

(i) Toxin neutralization on cell cultures
Provide separately all relevant information such as identity and titre (in IU/ml) of the reference vaccine, species of animals immunized, dilutions of vaccines, date of immunization, date of bleeding, dilution at which immune sera were assayed, amount of toxin added to dilutions of immune sera and, for each dilution: No. of wells (or tubes) where cells survived No. of wells inoculated with toxin–antitoxin mixtures or (control) toxin, median effective doses (ED_{50}s), titre per single human dose, limits of 95% confidence intervals, and any other relevant information.

(ii) Other test, e.g., competitive enzyme-linked immunosorbent assay (ELISA)
Provide separately all relevant information, including the data by which the method was validated.

Potency of test vaccine ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

Test for residual free detoxifying agent

Detoxifying agent (formaldehyde or glutaraldehyde) .....

Date of test .....

Result (in g/l) .....

pH

Date of measurement .....

Result .....

Final product

Identity test

Date of test .....

Type of test and result .....

Sterility test

No. of times the test had to be performed .....

No. of containers tested .....

Media and temperatures of incubation .....

Date of inoculation .....

Date of end of observation .....

Result of last test .....

Potency test

If this test was not performed on the final bulk, indicate this and report the data obtained on the final product in the space provided for potency tests in the "final bulk" section.

Innocuity test	*Mice*	*Guinea-pigs*
No. of animals	.....	.....
Route of injection	.....	.....
Volume of injection	.....	.....
Date of start of test	.....	.....
Date of end of test	.....	.....
Results	.....	.....

Test for adjuvant

Date of test .....

Nature and concentration of adjuvant per single human dose .....

Test for preservative

Date of test .....

Nature and concentration of preservative .....

pH

Date of measurement .....

Result .....

Inspection of final containers

Date of inspection .....

Result .....

Stability test[1]

Indicate separately all relevant details and (as a percentage) the calculated losses of potency per year and half-lives at different temperatures as determined by accelerated degradation tests, and actual titres[2] (with 95% limits of confidence intervals) after storage for the maximum period claimed for the product at the recommended temperature.

Certification by the manufacturer

Name of head of production (typed) .....

Certification by person from the control laboratory of the manufacturing company taking overall responsibility for the production and control of the vaccine

I certify that lot No. ... of diphtheria vaccine (adsorbed), whose number appears on the label of the final containers, meets all national requirements[3] and satisfies Part A of the diphtheria vaccine section of Requirements for Biological Substances Nos. 8 and 10, revised 1989 and (if applicable) addenda 19...

Signature .....

Name (typed) .....

Date .....

Certification by the national control authority

If the vaccine is to be exported, attach a certificate from the national control authority, a model of which is shown in Appendix 2, a label from a final container, and an instruction leaflet for users.

---

[1] Not required in summary protocols of every batch.

[2] Needed only for three batches to validate the production method.

[3] If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot has nevertheless been authorized.

Appendix 2

MODEL CERTIFICATE FOR THE RELEASE OF VACCINES

This certificate is to be provided by the national control authority of the country where the vaccines have been manufactured, upon request by the manufacturer

The following lots of .....[1] vaccine produced by .....[2] in .....,[3] whose numbers appear on the labels of the final containers, meet all national requirements,[4] Part A of the .....[1] section of the Requirements for Biological Substances Nos. 8 and 10 (Requirements for Diphtheria, Tetanus, Pertussis and Combined Vaccines, revised 1989 [if applicable, addendum 19...])[5] and the Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (revised 1965).[6]

Lot No.	Date of the last potency test by the manufacturer	Expiry date	Lot No.	Date of the last potency test by the manufacturer	Expiry date
.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	.....	.....
.....	.....	.....	.....	.....	.....

As a minimum, this certificate is based on an examination of the manufacturing protocol.

The number of this certificate is .....

The Director of the National Control Laboratory (or Authority as appropriate)[7]

Name (typed) .....

Signature .....

Date .....

---

[1] Indicate type of vaccine (e.g., tetanus, diphtheria–tetanus, diphtheria–tetanus–pertussis).

[2] Name of manufacturer.

[3] Country.

[4] If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national control authority.

[5] With the exception of the provisions on shipping, which the national control authority may not be in a position to control.

[6] Published in WHO Technical Report Series, No. 323, 1966.

[7] Or his or her representative.

Appendix 3

SUMMARY PROTOCOL FOR TETANUS VACCINE (ADSORBED) PRODUCTION AND TESTING

Summary information on final lot

Name and address of manufacturer .....

.....

.....

Lot No. .....

Date of filling .....

Volume of each recommended single human dose .....

No. of doses per final container .....

No. of final containers .....

Expiry date .....

Detailed information on manufacture and control

Strain

Identity of *C. tetani* strain used for vaccine production .....

Reference No. of seed lot .....

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

Single harvests used for preparing the bulk purified toxoid

List the single harvests and indicate the medium, dates of inoculation, temperature of incubation, dates of harvests, volumes, results of tests for bacterial purity, method of inactivation and yields.

Bulk purified toxoid

Reference No. .....

Volume and Lf/ml .....

Date and result of test for antigenic purity (Lf/mg of protein nitrogen) .....

Test of irreversibility

Lf/ml of test toxoid solution .....

Temperature of incubation of test toxoid .....

Dates of beginning and end of incubation .....

No. of guinea-pigs injected, route and date of injection .....

Date of end of observation .....

Result of test .....

Specific toxicity test

No. of guinea-pigs injected and date of injection .....

No. of Lf per guinea-pig and route of injection .....

Date of end of observation .....

Result of test .....

Final bulk

Identification .....

Volume .....

Lf/ml .....

Sterility test

Date and result of test .....

Specific toxicity test (optional)

No. of guinea-pigs injected and date of injection .....

Volume and route of injection .....

Date of end of observation .....

Result of test .....

Potency test[1]

(1) Based on lethal or paralytic challenge

(i) Three-dilution assays

Species and weight of animals .....

Date of immunization and volume of dilutions administered .....

Date of challenge .....

Challenge dose (indicate whether lethal or paralytic) .....

[1] Only one of the potency tests listed need be performed.

Date of end of observation .....

Results

	Dilution	*No. of survivors (or of animals not paralysed)/No. of animals injected*	*Median effective dose (ED_{50})*
Reference vaccine (... IU/ml)	.....	.....	
	.....	.....	..... ml
	.....	.....	
Test vaccine	.....	.....	
	.....	.....	..... ml
	.....	.....	

Potency of test vaccine is ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

(ii) One-dilution challenge test

Date of performance of last satisfactory three-dilution test .....

Nature and reference No. of product tested (specify also whether it was a final bulk or a final product) .....

Provide relevant information validating the one-dilution assay system.

Identity and titre (IU/ml) of reference vaccine .....

Animal species and weight of animals .....

Date of immunization .....

Date of challenge .....

Challenge dose (specify whether lethal or paralytic) .....

Date of end of observation .....

Results

	Reference vaccine	*Test vaccine*
Dilution used for immunization	.....	.....
No. of survivors (or of animals not paralysed)/No. injected	.....	.....

P value indicating the probability that the test vaccine contains more than 40 IU/ single human dose .....

(2) Test based on measuring antitoxin induction as an alternative to lethal or intradermal challenge

Provide separately all relevant information on other tests, e.g., competitive enzyme-linked immunosorbent assay (ELISA) or passive haemagglutination, including the data by which the method was validated.

Potency of test vaccine ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

Test for residual free detoxifying agent

Detoxifying agent (formaldehyde or glutaraldehyde) .....

Date of test .....

Result (in g/l) .....

pH

Date of measurement .....

Result .....

Final product

Identity test

Date of test .....

Type of test and result .....

Sterility test

No. of times the test had to be performed .....

No. of containers tested .....

Media and temperatures of incubation .....

Date of inoculation .....

Date of end of observation .....

Result of last test .....

Potency test

If the test was not performed on the final bulk, indicate this and report the data in the space provided for potency tests in the "final bulk" section.

Innocuity test	*Mice*	*Guinea-pigs*
No. of animals	.....	.....
Route of injection	.....	.....

	Mice	*Guinea-pigs*
Volume of injection	.....	.....
Date of start of test	.....	.....
Date of end of test	.....	.....
Results	.....	.....

Test for adjuvant

Date of test .....

Nature and concentration of adjuvant per single human dose .....

Test for preservative

Date of test .....

Nature and concentration of preservative .....

pH

Date of measurement .....

Result .....

Inspection of final containers

Date of inspection .....

Result .....

Stability test[1]

Indicate separately all relevant details and (as a percentage) the calculated losses of potency per year at different temperatures as determined by accelerated degradation tests, and actual titres[2] (with limits of 95% confidence intervals) after storage for the maximum period claimed for the product at the recommended temperature.

[1] Not required in summary protocols of every batch.

[2] Needed only for three batches to validate the production method.

Certification by the manufacturer

Name of head of production (typed) .....

Certification by person from the control laboratory of the manufacturing company taking overall responsibility for the production and control of the vaccine

I certify that lot No. ... of tetanus vaccine (adsorbed), whose number appears on the label of the final containers, meets all national requirements[1] and satisfies Part A of the tetanus vaccine section of Requirements for Biological Substances Nos. 8 and 10, revised 1989 and (if applicable) addenda 19...

Signature .....

Name (typed) .....

Date .....

Certification by the national control authority

If the vaccine is to be exported, attach a certificate from the national control authority as shown in Appendix 2, a label from a final container and an instruction leaflet for users.

[1] If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot has nevertheless been authorized.

Appendix 4

SUMMARY PROTOCOL FOR PERTUSSIS VACCINE PRODUCTION AND TESTING

Summary information on final lot

Name and address of manufacturer .....
.....
.....

Lot No. .....

Date of filling .....

Nature of final product (plain or absorbed) .....

Volume of each recommended single human dose .....

No. of doses per final container .....

No. of final containers .....

Expiry date .....

Detailed information on manufacture and control

Strain

Identity of *B. pertussis* strains used in vaccine .....

Serological types of strains .....

Reference No. of seed lot .....

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

Single harvests used for preparing final bulk

List the single harvests and indicate the medium, dates of inoculation, temperature of incubation, dates of harvests, volumes, results of tests for bacterial purity, methods and dates of inactivation, opacity, and agglutinogens present.

Final bulk

Identification .....

Volume .....

No. of opacity units (calculated from opacities of single harvests) .....

Test for agglutinogens 1, 2 and 3

Date and results (before addition of adjuvant) .....

Sterility test

Medium, date and result of test .....

Specific toxicity test (mouse weight-gain test)

Strain of mice .....

No. of animals in test group and control group .....

Date of injection .....

Volume and route of injection .....

Date of end of observation .....

Result of test: on a separate sheet of paper, give all relevant details for mice in the control and test groups (survival, mean weight on day of injection and three and seven days after it) and indicate percentage weight of test group as compared with control group.

Other specific toxicity tests

Mention here date and results of any other specific toxicity test which may have been performed (e.g., tests for heat-labile toxin, lymphocytosis promoting factor and endotoxin) .....

Potency test

Strain, weight and sex of mice .....

Date of immunization .....

LD_{50} in challenge dose .....

No. of colony-forming units in challenge dose .....

Date of challenge .....

Date of end of observation .....

Results .....

	Dilution	*No. of survivors/ No. inoculated*	*Median effective dose (ED_{50})*
Reference vaccine (... IU/ml)	.....	.....	..... ml
	.....	.....	
	.....	.....	

	Dilution	*No. of survivors/ No. inoculated*	*Median effective dose (ED_{50})*
Test vaccine	.....	.....	
	.....	.....	..... ml
	.....	.....	

Potency of test vaccine is ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

pH

Date of measurement .....

Result .....

Final product

Identity test

Date of test .....

Type of test and result .....

Sterility test

No. of times the test had to be performed .....

No. of containers tested in each test .....

Media and temperatures of incubation .....

Date(s) of inoculation .....

Date(s) of end of observation .....

Result of each test .....

Potency test

If the test was not performed on the final bulk, indicate this and report the data obtained on the final product in the space provided for potency tests in the "final bulk" section.

Innocuity test	Mice	Guinea-pigs
No. of animals	.....	.....
Route of injection	.....	.....
Volume of injection	.....	.....
Date of start of test	.....	.....
Date of end of test	.....	.....
Results	.....	.....

Test for adjuvant

Date of test .....

Nature and concentration of adjuvant per single human dose .....

Test for preservative

Date of test .....

Nature and concentration of preservative .....

pH

Date of measurement .....

Result .....

Inspection of final containers

Date of inspection .....

Result .....

Stability test[1]

Indicate separately all relevant details and (as a percentage) the calculated losses of potency per year at different temperatures, as determined by accelerated degradation tests, and actual titres[2] (with limits of 95% confidence intervals) after storage for the maximum period claimed for the product at the recommended temperature.

Certification by the manufacturer

Name of head of production (typed) .....

Certification by person from the control laboratory of the manufacturing company taking overall responsibility for the production and control of the vaccine

I certify that lot No. ... of pertussis vaccine, whose number appears on the label of the final containers, meets all national requirements[3] and satisfies Part A of the pertussis vaccine section of Requirements for Biological Substances Nos. 8 and 10, revised 1989 and (if applicable) addenda 19...

Signature .....

Name (typed) .....

Date .....

Certification by the national control authority

If the vaccine is to be exported, attach a certificate from the national control authority as shown in Appendix 2, a label from a final container, and an instruction leaflet for users.

---

[1] Not required in summary protocols of every batch.

[2] Needed only for three batches to validate the production method.

[3] If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot has nevertheless been authorized.

Appendix 5

SUMMARY PROTOCOL FOR DIPHTHERIA, TETANUS AND PERTUSSIS VACCINE (ADSORBED) PRODUCTION AND TESTING[1]

Summary information on final lot

Name and address of manufacturer .....

.....

.....

Lot No. .....

Date of filling .....

Volume of each recommended single human dose .....

No. of doses per final container .....

No. of final containers .....

Expiry date .....

Detailed information on manufacture and control

Strains, single harvests, bulks

Diphtheria vaccine

Strain

Identity of *C. diphtheriae* strain used for vaccine production .....

Reference No. of seed lot .....

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

Single harvests used for preparing bulk purified toxoid

List the single harvests and indicate the medium, dates of inoculation, temperature of incubation, dates of harvests, volumes, results of tests for bacterial purity, method of inactivation and yields.

---

[1] For diphtheria–tetanus vaccines, delete "Pertussis" in the title of the protocol and do not fill in sections relating solely to pertussis vaccine.

Bulk purified toxoid

Reference No. .....

Volume and Lf/ml .....

Date and result of test for antigenic purity (Lf/mg of protein nitrogen) .....

Test of irreversibility

Lf/ml of test toxoid solution .....

Temperature of incubation of test toxoid .....

Dates of beginning and end of incubation .....

No. of guinea-pigs injected, route and date of injection[1] .....

Date of end of observation .....

Result of test .....

Specific toxicity test[1]

No. of guinea-pigs injected and date of injection .....

No. of Lf per guinea-pig and route of injection .....

Date of end of observation .....

Result of test .....

Tetanus vaccine

Strain

Identity of *C. tetani* strain used for vaccine production .....

Reference No. of seed lot .....

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

Single harvests used for preparing bulk purified toxoid

List the single harvests and indicate the medium, dates of inoculation, temperature of incubation, dates of harvests, volumes, results of tests for bacterial purity, yields and method of inactivation.

[1] If a cell-culture system was used, provide all appropriate information.

Bulk purified toxoid

Reference No. .....

Volume and Lf/ml .....

Date and result of test for antigenic purity (Lf/mg of protein nitrogen) .....

Test of irreversibility

Lf/ml of test toxoid solution .....

Temperature of incubation of test toxoid .....

Dates of beginning and end of incubation .....

No. of guinea-pigs injected, route and date of injection .....

Date of end of observation .....

Result of test .....

Specific toxicity test

No. of guinea-pigs injected and date of injection .....

No. of Lf per guinea-pig and route of injection .....

Date of end of observation .....

Result of test .....

Pertussis vaccine

Strain

Identity of *B. pertussis* strain used in vaccine .....

Serological type of strain .....

Reference No. of seed lot .....

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

Single harvests used for preparing the bulk material

List the single harvests and indicate the medium, dates of inoculation, temperature of incubation, dates of harvests, volumes, results of tests for bacterial purity and presence of agglutinogen, methods and dates of inactivation, and opacity.

Bulk material

Identification .....

Volume and opacity/ml .....

Date, results of, and medium used in test for living organisms .....

Date of test for presence of agglutinogens 1, 2 and 3 and results .....

Information on blending

Diphtheria toxoid component

Reference No. .....

Lf/ml .....

Volume .....

Tetanus toxoid component

Reference No. .....

Lf/ml .....

Volume .....

Pertussis vaccine component

Reference No. .....

Opacity units (calculated from opacities of single harvests) .....

Volume .....

Adjuvant

Nature and concentration (Al or Ca in mg/ml) .....

Volume .....

Preservatives

Nature and concentration .....

Volume .....

Buffer

Nature and concentration .....

Volume .....

Tests on final bulk

Reference No. .....

Date of completion .....

Volume .....

Sterility test

Date and result of test .....

Specific toxicity test

Tetanus and diphtheria (optional)

No. of guinea-pigs injected and date of injection .....

Number of single human doses injected per guinea-pig, volume and route of injection .....

Date of end of observation .....

Result of test .....

Pertussis

(i) Mouse weight-gain test
Strain of mice .....

No. of animals in test group and control group .....

Date of injection .....

Volume and route of injection .....

Date of end of observation .....

Results of test: on a separate sheet of paper, give all relevant details for mice in the control and test groups (survival, mean weight before injection and three and seven days after it) and indicate percentage weight gain of test group as compared with control group.

(ii) Other tests
Mention here date and results of any other specific toxicity test which may have been performed (e.g., tests for heat-labile toxin, lymphocytosis promoting factor, pertussis toxin on cell cultures and endotoxin).

Potency test

Diphtheria[1]

(i) Tests based on challenge
(*a*) Three-dilution assays

Lethal challenge

Weight of guinea-pigs .....

Date of immunization and volume of dilutions administered .....

Date of challenge .....

[1] Only one of the potency tests described here need be performed.

Challenge dose .....

Date of end of observation .....

Results

	Dilution	*No. of survivors/No. of animals injected*	*Median effective dose (ED_{50})*
Reference vaccine (... IU/ml)	.....	.....	..... ml
	.....	.....	
	.....	.....	
Test vaccine	.....	.....	
	.....	.....	..... ml
	.....	.....	

Potency of test vaccine is ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

Multiple intradermal challenge

Report all relevant information on animals, and dates of immunization, challenge, and end of observation

Results

	Dilution	*Mean score*
Reference vaccine (... IU/ml)	.....	.....
	.....	.....
	.....	.....
Test vaccine	.....	.....
	.....	.....
	.....	.....

Potency of test vaccine is ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

(*b*) One-dilution challenge test

Date of performance of last satisfactory three-dilution test .....

Nature and reference No. of product tested (specify also whether it was a final bulk or a final product) .....

Provide relevant information validating the one-dilution assay system.

Identity and titre (IU/ml) of reference toxoid .....

Weight of guinea-pigs .....

Date of immunization .....

Date of challenge .....

Challenge dose .....

Date of end of observation .....

Results

	Reference vaccine	*Test vaccine*
Dilution used for immunization	.....	.....
No. of survivors/No. injected	.....	.....

P value indicating the probability that the test vaccine contains more than 30 IU per single human dose .....

(ii) Tests not based on challenge

(*a*) Toxin neutralization on cell cultures

Provide separately all relevant information such as identity and titre (in IU/ml) of the reference vaccine, species of animals immunized, dilutions of vaccines, date of immunization, date of bleeding, dilution at which immune sera were assayed, amount of toxin added to dilutions of immune sera and, for each dilution: No. of wells (or tubes) where cells survived/No. of wells inoculated with toxin–antitoxin mixtures or (control) toxin, median effective doses (ED_{50}s), titre per single human dose, confidence interval, etc.

(*b*) Other tests, e.g., competitive enzyme-linked immunosorbent assay (ELISA)

Provide separately all relevant information, including data by which the method was validated.

Potency of test vaccine ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

Tetanus[1]

(i) Tests based on lethal or paralytic challenge

(*a*) Three-dilution assays

Species and weight of animals .....

Date of immunization and volume of dilutions administered .....

Date of challenge .....

Challenge dose (indicate whether lethal or paralytic) .....

Date of end of observation .....

[1] Only one of the potency tests listed here need be performed.

Results

	Dilution	*No. of survivors (or of animals not paralysed)/No. of animals injected*	*Median effective dose (ED_{50})*
Reference vaccine (... IU/ml)	.....	.....	
	.....	.....	..... ml
	.....	.....	
Test vaccine	.....	.....	
	.....	.....	..... ml
	.....	.....	

Potency of test vaccine is ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

(*b*) One-dilution challenge test

Date of performance of last satisfactory three-dilution test .....

Nature and reference No. of product tested (specify also whether it was a final bulk or a final product) .....

Provide relevant information validating the one-dilution assay system.

Identity and titre (IU/ml) of reference toxoid .....

Animal species and weight of animals .....

Date of immunization .....

Date of challenge .....

Challenge dose (specify whether lethal or paralytic) .....

Date of end of observation .....

Results

	Reference vaccine	*Test vaccine*
Dilution used for immunization	.....	.....
No. of survivors (or of animals not paralysed)/No. injected	.....	.....

P value indicating the probability that the test vaccine contains more than 40 IU/single dose (60 IU if DTP vaccine is assayed in mice) .....

(ii) Tests not based on challenge

Provide separately all relevant information on other tests, e.g., competitive enzyme-linked immunosorbent assay (ELISA) or passive haemagglutination, including the data by which the method was validated.

Potency of test toxoid is ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

Pertussis

Strain, weight and sex of mice .....

Date of immunization .....

LD_{50} in challenge dose .....

No. of colony-forming units in challenge dose .....

Date of challenge .....

Date of end of observation .....

Results

	Dilution	*No. of survivors/ No. inoculated*	*Median effective dose (ED_{50})*
Reference vaccine (... IU/ml)	.....	.....	
	.....	.....	..... ml
	.....	.....	
Test vaccine	.....	.....	
	.....	.....	..... ml
	.....	.....	

Potency of test vaccine is ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

Test for residual free detoxifying agent

Detoxifying agent (formaldehyde or glutaraldehyde) .....

Date of test .....

Result (in g/l) .....

pH

Date of measurement .....

Result .....

Tests on final product

Identity test

Test for diphtheria toxoid: method, date and results .....

Test for tetanus toxoid: method, date and results .....

Test for pertussis vaccine: method, date and results .....

Sterility test

No. of times the test had to be performed .....

No. of containers tested .....

Media and temperatures of incubation .....

Date of inoculation .....

Date of end of observation .....

Result of the (last) test .....

Potency test

If this test was not performed on the final bulk, indicate this and report the data obtained on the final product in the space provided for potency tests in the "final bulk" section.

Innocuity test	*Mice*	*Guinea-pigs*
No. of animals	.....	.....
Route of injection	.....	.....
Volume of injection	.....	.....
Date of injection	.....	.....
Date of end of observation	.....	.....
Results	.....	.....

Test for adjuvant

Date of test .....

Nature and concentration of adjuvant per single human dose .....

Test for preservative

Date of test .....

Nature and concentration of preservative .....

pH

Date of measurement .....

Result .....

Inspection of final containers

Date of inspection .....

Result .....

Stability test[1]

For each component of the vaccine, indicate separately all relevant details and (as a percentage) the calculated losses of potency per year at different temperatures as determined by accelerated degradation tests, and actual titres[2] (with the limits of 95% confidence intervals) after storage for the maximum period claimed for the product at the recommended temperature.

Certification by the manufacturer

Name of head of production (typed) .....

Certification by person from the control laboratory of the manufacturing company taking overall responsibility for the production and control of the vaccine

I certify that lot No. ... of diphtheria, tetanus and pertussis vaccine, whose number appears on the label of the final containers, meets all national requirements[3] and satisfies Part A of the combined vaccines section of Requirements for Biological Substances Nos. 8 and 10, revised 1989 and (if applicable) addenda 19...

Signature .....

Name (typed) .....

Date .....

Certification by the national control authority

If the vaccine is to be exported, attach a certificate from the national control authority as shown in Appendix 2, a label from a final container, and an instruction leaflet for users.

---

[1] Not required in summary protocols of every batch.

[2] Needed only for three batches to validate the production method.

[3] If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot has nevertheless been authorized.

World Health Organization, Technical Report Series, No. 800, 1990

Annex 3

REQUIREMENTS FOR ANTIMICROBIC SUSCEPTIBILITY TESTS 1. AGAR DIFFUSION TESTS USING ANTIMICROBIC SUSCEPTIBILITY DISCS

(Requirements for Biological Substances No. 26)
(Revised 1981[1], Addenda 1982[2], 1985[3], 1987[4])

Addendum 1989

As part of these Requirements, the Expert Committee on Biological Standardization at its thirty-third meeting[2] adopted a revised list of the codes used to identify antimicrobials contained in susceptibility test discs. The list of codes was again revised at subsequent meetings of the Expert Committee to incorporate additions, deletions and changes in nomenclature. During the period since the last revision, further requests have been received by the WHO Secretariat for the allocation of codes for new antimicrobial substances. In order to incorporate the new entries that have been agreed, the following further additions should be made to the list of codes given in Part A, Section 1.6 of the Requirements.[2]

Add:

cefodizime	CDZ
cefpimizole	CFZ
cefpirome	CPO
cefpodoxime	CPD
lomefloxacin	LOM
meropenem	MEM

[1] WHO Technical Report Series, No. 673, 1982.
[2] WHO Technical Report Series, No. 687, 1983.
[3] WHO Technical Report Series, No. 745, 1987.
[4] WHO Technical Report Series, No. 771, 1988.

World Health Organization, Technical Report Series, No. 800, 1990

Annex 4

GUIDELINES FOR THE PREPARATION, CHARACTERIZATION AND ESTABLISHMENT OF INTERNATIONAL AND OTHER STANDARDS AND REFERENCE REAGENTS FOR BIOLOGICAL SUBSTANCES

(Revised 1989)

INTRODUCTION

In 1978, WHO published the Guidelines for the Preparation and Establishment of Reference Materials for Biological Substances (*1*). Since then, the WHO Expert Committee on Biological Standardization has discussed the content and implications of the Guidelines on several occasions. In particular, in 1983, the Committee agreed that it was no longer appropriate for international reference preparations to which an activity expressed in International Units has been assigned to constitute a separate category (*2*). In addition, in 1985 the Committee discussed issues relating to the safe handling of reference materials of human origin, and requested the WHO Secretariat to amend the Guidelines by introducing the testing of certain proposed reference materials for evidence of possible contamination with human immunodeficiency viruses and hepatitis B virus (*3*).

These two considerations led to the revision of the Guidelines in 1986 (*4*). In 1988, at its thirty-ninth meeting (*5*), the Expert Committee on Biological Standardization recommended that the WHO Secretariat should again revise the Guidelines to bring the presentation into line with that used for the Requirements on Biological Substances, for example by putting parts of the text in large and others in small print, depending on their importance. Accordingly those parts of these revised Guidelines considered to be important are printed in large type, whereas comments or recommendations have been printed in small type. In addition, a section has been included on information to be provided to Biologicals, WHO, in support of the submission of requests for adoption by the WHO Expert Committee on Biological Standardization of biological substances as international reference materials. In the process of revising the Guidelines, account has also been taken of several suggestions as to ways in which they could be improved.

The advice provided by a number of scientists during the preparation of these revised Guidelines is gratefully acknowledged (see p. 212).

Part A of these Guidelines has been formulated so that international associations and groups of experts, which to an increasing extent are making proposals for, and becoming involved in, the preparation and testing of international biological reference materials, may become familiar with the procedures followed for the establishment of such materials.

Part B of these Guidelines has been formulated with the aim of assisting national control authorities and individual laboratories seeking guidance on the preparation and establishment of national or laboratory working standards, in particular by indicating how procedures in general use differ from those appropriate to the establishment of international biological reference materials.

PART A. GUIDELINES FOR THE PREPARATION, CHARACTERIZATION AND ESTABLISHMENT OF INTERNATIONAL BIOLOGICAL STANDARDS AND REFERENCE REAGENTS

1. Introduction

1.1 Background

WHO establishes international biological standards and reference reagents for substances of biological or synthetic origin that cannot be characterized adequately by chemical and/or physical means alone and that are used in the prophylaxis, therapy or diagnosis of human and certain animal diseases. International biological standards are established primarily to enable the activity of biological preparations to be expressed in the same way, generally in International Units, throughout the world. The function of reference reagents is either to provide a means of checking the specificity of biological diagnostic reagents, or to enable certain preparations of biological substances to be calibrated in a system that differs from the international unitage system.

International biological standards and reference reagents are not necessarily of high purity. They are not generally available in sufficient quantity for routine laboratory use.

1.2 Definition of international biological standards and reference reagents

An *international biological standard* is a preparation of a substance of biological or synthetic origin by means of which the World Health Organization generally defines an international unit after an international study has been completed. An international

unitage is usually attributed to a first international standard in an arbitrary manner. In contrast, activities in International Units are assigned to replacement international standards by calibrating them against the previous standards in international collaborative studies. In all cases, such studies must show that the proposed materials are suitable to serve as international standards.

International biological reference reagents are used, for example, for the identification of microorganisms (or products derived from them) or for the diagnosis of diseases. They were originally established to provide a means of ensuring the specificity of the corresponding working reagents. However, certain reference reagents (e.g., the international reference reagents for the titration of measles vaccines or for the assay of toxoids by flocculation) are used in quantitative assays in which it is inappropriate to express the activity of the biological products tested in International Units. In such cases, units of activity may be assigned to reference reagents in terms of a suitable property, such as the reciprocal of the dilution at which a particular end-point occurs. The distinction between international standards and international reference reagents is based, *inter alia*, on the purpose for which they are used and the extent of their characterization. However, the distinction is not always clear-cut.

1.3 Purpose of the guidelines on international reference materials

Four centres designated by WHO as International Laboratories for Biological Standards are the custodians of international biological standards and reference reagents (*6*). In the past, substances serving as international biological standards have been prepared largely by these international laboratories or by other laboratories in direct collaboration with them. In recent years, however, there has been a considerable expansion of the work undertaken in biological standardization.

> This has led to a number of laboratories and institutions becoming involved in making preparations that may be intended in the first instance for field trials or laboratory studies, but are ultimately offered to WHO for consideration as international biological standards or reference reagents.

At its twenty-seventh meeting, the Expert Committee on Biological Standardization recommended that, in future, all proposals by international associations for the establishment of

international biological standards and reference reagents should be channelled through WHO so as to avoid duplication of effort (*7*). The Committee considered that priority should be given to the standardization of substances used in the prophylaxis, therapy or diagnosis of diseases and for which, by the use of international standards, it would be possible to compare activities anywhere in the world.

> The Expert Committee on Biological Standardization does not normally concern itself with the establishment of standards or reference reagents for biological substances used only for research purposes.

This part of the Guidelines has been drawn up so that international associations and groups of experts, which are to an increasing extent making proposals for, and becoming involved in, the preparation and testing of international biological reference materials, may become familiar with the procedure followed in the preparation, characterization and establishment of such materials.

1.4 Safety considerations

For safety reasons, biological materials of human origin that are considered for the preparation either of international standards or of reference reagents should be tested for the presence of hepatitis B surface antigen, antibodies to human immunodeficiency viruses (HIV) and (when appropriate tests are available) HIV antigens, as well as for other relevant pathogens. Tests for the presence of HIV markers may not be required for reference materials intended for the diagnosis of HIV infections; in such cases, suitable evidence of proper inactivation should be provided. When tests for the presence of certain suspected pathogens (e.g., the "slow virus" associated with Creutzfeldt–Jakob disease) in a given biological material are not available, alternative (e.g., recombinant DNA-derived) sources of material should be sought, provided that the material satisfies the criteria applicable to its use as international reference material. In all cases, suitable precautions in the handling and disposal of biological materials should be taken to avoid possible infection.

2. Assessment of need and procurement of materials

2.1 Assessment of need

International biological reference materials may be needed for:

(1) the assay of any biological product used in medical or veterinary practice and distributed in more than one country,
(2) the assay of a new biological product that has a clear potential for use and distribution in more than one country,
(3) the calibration of national biological reference materials used widely in diagnostic tests, and
(4) the comparison of research data relating to biological materials requiring assay in the fields of the prevention, treatment and diagnosis of disease.

The evaluation of the need for international reference materials for the fourth of the uses listed above may be the most difficult. International scientific associations can be influential in making reference materials available before the need for corresponding international reference materials is apparent.

When a need for an international reference material is identified by an independent scientific body, it is essential that WHO should be informed whether that body intends to proceed with its preparation, so as to avoid unnecessary duplication of effort.

Where a case for establishing an international reference material with a defined unit of activity is made, a technique should exist whereby the activity can be measured, and this should preferably have been published. In many cases, a variety of assay methods will be available, and the material chosen should ideally be suitable for use with as many of them as possible.

2.2 Nature, source and storage of bulk material

The composition of a biological reference material should resemble as closely as possible that of the samples that will be assayed against it. The bulk material selected should have a high degree of stability and a specific activity sufficient for the purposes of the assays or tests for which it is intended.

The purity of the material should be such that no substances are present that would interfere with the procedures by which the material is to be tested, but it should be noted that the purest material is not necessarily the most suitable. Less pure forms

may be preferable if they are more stable or if the pure form is otherwise unsuitable. The bulk material is usually obtained from a single source and prepared by personnel experienced in handling the substance.

Bulk material may consist of part or all of a single batch or, if necessary, it may be prepared by pooling a number of different batches. If it is necessary to blend material from several sources, it should be recognized that the properties of the material may be affected.

When the bulk ingredients used in the preparation of international reference materials (or the final containers to be established as international reference materials) come from a commercial source, this fact should not be used for advertising purposes.

When different batches are pooled, the pooled material must be thoroughly mixed so as to ensure homogeneity. Irrespective of the studies that may have been made on the individual batches before pooling, the homogeneous bulk material itself should be studied for its suitability to serve as an international standard or reference reagent.

In general, to be suitable as an international standard, a material should be available in sufficient quantity to provide several (e.g., 10) years' supply. Small quantities of materials will be adequate only if the materials concerned are to be issued to a few laboratories only.

It is normal practice for each ampoule to contain enough material for several assays. However, if, after reconstitution of a lyophilized material, the solution is not stable, it may be desirable to subdivide the bulk into a larger number of containers, each sufficient for only one or two assays. For scarce materials, the decision as to the amount to be introduced into each ampoule should take into account the need to conserve the material.

If the bulk material has to be stored before being distributed into ampoules, it is essential that it is held under suitable conditions.

It is considered better to store bulk materials in the dried form, particularly if they are to be stored for long periods, provided that they can be dried without losing their biological activity and that, on being reconstituted, they are still of adequate activity and have appropriate physical properties.

Powders such as antibiotics can be stored successfully for long periods in screw-capped bottles in sealed polyethylene bags containing silica gel, in the dark and at low temperature, or in air-evacuated desiccators in the dark at $-10\,^{\circ}C$. Sera may usually be stored at 2–10 °C provided that they are sterile or contain an antimicrobial substance. However, it is advisable to

store certain sera in the frozen state, in which case special precautions should be taken to achieve proper freezing. In all cases, the containers should be able to tolerate the recommended conditions of freezing, storage, thawing, opening and, if applicable, freeze-drying.

Advice on optimum storage conditions should be sought from the producers of the materials before receipt of the batch. Before storage of the bulk material, sufficient samples should be removed to provide for all necessary testing. Until they are tested, these samples should be stored under the same conditions as the bulk.

3. Distribution into final containers

3.1 General considerations

An important requirement to be met by any batch of an international reference material is that the material in every ampoule in the batch should be identical in terms of composition, quantity, potency and stability.

The bulk material, which may be in the liquid or solid form, is distributed into a number of suitable containers so as to enable identical samples to be supplied to user laboratories without affecting the integrity or stability of the preparation. If all the samples of any one preparation are to form a homogeneous collection, they should all be derived from the same homogeneous bulk, and should all be processed together in one working session.

If the material is a liquid or if it is water-soluble, distribution in ampoules in the liquid form is to be preferred because of the ease of dispensing a liquid with the desired high degree of precision. Materials in liquid form that can be dried should be dried from the frozen state, possibly followed by secondary drying to reduce the solvent content still further. Such a process may also be applied to insoluble solids that can be suspended in a suitable liquid. Storage in an evacuated desiccator containing active phosphorus pentoxide under vacuum is a suitable method of secondary drying.

Materials in liquid form that cannot be dried satisfactorily may, after dispensing, be stored as liquids provided that stability is retained under the storage conditions that can be provided.

Suitable precautions should be taken to protect personnel if there is any possibility that the material contains contaminating organisms pathogenic to humans or is itself harmful.

3.2 Treatment of liquid bulk materials

The choice of process and the extent of processing required for preparing the final bulk for filling will depend on the nature of the liquid bulk, i.e., on whether it is a true solution, a colloid, or a suspension. In all cases the processing shall ensure that the product is homogeneous during filling, and measures should be taken at all stages to avoid contamination of the material.

Certain materials may have to be treated chemically or physically to control microbial contamination or to remove particles or aggregates of active material. Pyrogen-free double glass-distilled water should be used for the preparation of diluents or stabilizing solutions.

When an antimicrobial preservative is used, it should be one that will not affect the preparation during the drying process or its stability subsequently, and will not volatilize during drying. Thiomersal at a concentration of 0.1 g/l has been used successfully in many sera, in inactivated bacterial vaccines and in some inactivated viral vaccines. Cresol, phenol or sodium azide should not be used in a preparation that is to be freeze-dried.

A biologically active substance is frequently ampouled in such small amounts that a large amount of a bulking agent is added before ampouling. This allows a plug of suitable dimensions and visibility to be formed, thus aiding reconstitution. In some instances also, the added material will prevent or limit adsorption of the active substance on to the glass of the ampoule and other damage that may occur during freeze-drying.

Any added substance should be carefully chosen so that it has no deleterious effects on the activity of the material and does not interfere with the assay or test for which the preparation is intended.

A protein carrier such as human albumin is frequently used. It should be tested for freedom from contamination with proteolytic enzymes.

Recent experience has shown that certain polypeptides may combine irreversibly with substances such as lactose, mannitol and trehalose, commonly used as carriers in standards, to form complexes. It is therefore advisable to carry out preliminary freeze-drying trials, after which analytical methods such as high-performance liquid chromatography, polyacrylamide gel electrophoresis and isoelectric focusing are used in addition to assays of biological activity to determine whether the molecular integrity and purity of the sample have been preserved.

3.3 Treatment of solid bulk materials

Certain materials that are relatively insoluble in water or less stable in a freeze-dried form may have to be distributed into containers as powders.

If the material is to be distributed in the solid (powder) form, special precautions should be taken to ensure that both the bulk material and the samples taken from it are homogeneous, for which purpose the use of special mixing devices may be necessary.

3.4 Quality of final containers

It is the established practice to use heat-sealed ampoules in preference to rubber-stoppered vials for international reference materials to which units of activity are assigned since, in a sealed glass ampoule, there is virtually no exchange of gases and moisture with the atmosphere and the long-term stability of biological materials is generally much greater under these conditions.

Whereas final containers of international standards must consist of sealed ampoules made of neutral glass, the use of rubber-stoppered vials may be considered for the preparation of international reference reagents that are used for qualitative purposes, where stability considerations are less important.

Containers should be of neutral (borosilicate) glass type 1 of appropriate quality.[1] The glass must be free from stresses and be able to withstand both sterilization by heat and temperature stresses, such as those inherent in rapid freezing to −80 °C or below, to which the ampoule may be subjected. The walls of the ampoule should have a thickness of at least 0.5 mm and be impervious to gases.

The shape and size of the ampoules should be such that they can be filled easily, sealed by fusion of the glass without detrimental effect to the contents, and opened easily and their contents removed without difficulty. The volume of the ampoules to be selected depends on the amount of material required in each; a capacity of about 5 ml is generally suitable for fills up to 1.0 ml in volume. Actinic glass ampoules may be needed for photosensitive materials.

Whenever a preparation is to be lyophilized, it is advisable to use flat-bottomed ampoules in order to ensure good thermal

[1] Complying with the *European Pharmacopoeia*, 2nd ed. *Part I, section VI.2.1.* Sainte-Ruffine, Maisonneuve, 1980; or the *United States Pharmacopeia*, 21st revision. *Section 661.* Rockville, United States Pharmacopeial Convention, Inc., 1985, pp. 1233–1235.

conductivity between the bottom of the ampoule and the top of the shelf.

Batches of ampoules should be tested for conformity to specifications. The ampoules should be cleaned by heating in distilled water in an autoclave, by steaming in hydrochloric acid (20 g/l), or by ultrasonic treatment, followed by several rinses with clean water and finally with distilled water. Steam admitted to autoclaves for the cleaning or sterilization of glassware must be free from any volatile or nonvolatile compounds that may be present as a result of the use of boiler-water additives. If steaming in hydrochloric acid is carried out in an autoclave, great care must be taken to remove traces of the acid from the autoclave after the cleaning treatment. Detergents should not be used. The washed ampoules should then be sterilized by dry heat in a clean, grease-free and silicone-free oven. If the ampoules are to be stored at any stage after cleaning and before filling, they should be placed in sealed dustproof containers.

3.5 Distribution into ampoules

3.5.1 *General considerations*

Before filling, each ampoule should be permanently marked with some form of identification of the filling batch, e.g., by ceramic printing or etching or by printing on heat-sensitive labels, but not by scratching with a diamond point.

Ampoules should be filled from a single homogeneous bulk of material. To ensure that homogeneity is maintained throughout the filling process, the bulk material should be held at constant temperature during filling and, if necessary, stirred continuously. It should also be shielded from direct sunlight.

The filling should be carried out in such a way as to avoid any form of contamination (e.g., by microorganisms, chemicals or dust). This can be achieved by filling in a clean room or in a laminar-flow cabinet equipped with a HEPA filter.

3.5.2 *Liquid fills*

During any filling run, at least 1–2% of the ampoules should be selected and weighed before and after filling in order to check the variation in the amount of material from ampoule to ampoule (precision of fill or coefficient of variation). Such ampoules should be selected at regular intervals throughout the filling run.

When this is necessary for the purposes of the analysis, for ampoules containing liquids intended to serve as international

standards, a coefficient of variation (standard deviation divided by the mean) not greater than 0.0025 (i.e., 0.25%) is appropriate.

A larger variation may be tolerated when this is justified by the nature or intended use of the material.

3.5.3 *Powder fills*

Powder fills are used only occasionally.

In distributing solid materials, large variations in amounts per ampoule may be unavoidable. This may be unimportant, however, since an exact quantity of the contents can be weighed at the time of use. Most powders can be fed into ampoules by means of an automatic filler, but spoons of suitable size may also be used. In some cases, the powder may acquire an electrostatic charge and stick to the inside of the ampoule, and this may cause the charring of the adherent powder when the ampoule is sealed. In order to avoid this, it may be necessary to use special filling devices, e.g., a funnel with the stem reaching to about 10 mm from the bottom of the ampoule. During filling with certain substances, e.g., those that are hygroscopic or efflorescent, special precautions should be taken to control the atmospheric humidity.

4. Processing of filled ampoules

4.1 General considerations

The processing of filled ampoules should be completed with the minimum delay. It is essential to ensure that, from the time of filling up to the end of the operation, all the ampoules in a batch are processed together so that they are subjected to the same conditions at the same time. It has been shown that a small amount of cross-contamination can occur when different materials are freeze-dried together. Careful consideration must be given to this possibility and, wherever possible, only one material should be processed at a time in the freeze-drier.

In all cases, ampoules should be sealed by fusion of the glass, since sealing with rubber or plastic closures may be unsatisfactory in long-term storage.

It is desirable for samples to be taken so as to enable the "baseline" potency to be assessed at appropriate times during processing; they should be preserved over liquid nitrogen. This

makes it possible to evaluate the effects of processing on the biological material and ensures, for example, that there has been no loss of biological activity or that no aggregates have appeared.

4.2 Processing of materials that are to be freeze-dried

4.2.1 *Freezing*

The liquid in the ampoules should be frozen to well below the measured eutectic temperature, or to about $-60\,^{\circ}C$ if measurement of the eutectic temperature of the mixture is difficult or impossible. It is advisable for the temperature to be low enough to ensure complete freezing.

When a solution of sodium chloride is cooled slowly, ice crystals consisting of pure water will form. As these crystals grow, they exclude sodium chloride molecules until the latter reach a critical concentration called the "eutectic concentration" at which the whole mixture freezes solid; the temperature at which the solution freezes depends on its composition, even minor differences (especially in terms of small molecules in, say, different buffers) having a significant effect on it. When serum is cooled slowly below $0\,^{\circ}C$, crystals consisting initially of pure water form and grow. These crystals tend to exclude other molecules (such as electrolytes, sugars and proteins), which tend to become concentrated in pockets of solution between them. Depending on the rate of cooling and the temperature reached, the greatly increased salt concentration and associated pH changes may damage proteins and result in loss of their biological activity. Some proteins are more susceptible to such damage than others; certain antibodies, clotting factors and enzymes, for example, are notoriously liable to denaturation of this type. Although the freezing point of a eutectic solution of sodium chloride (the main electrolyte present in serum) is around $-22\,^{\circ}C$, serum does not appear to be "completely frozen" until around -50–$60\,^{\circ}C$, owing to the other small molecules present. Ideally, the temperature at which any given solution is completely frozen should be determined, e.g., by differential thermal analysis (which is suitable for simple solutions of electrolytes but not for complex solutions such as serum because the end-point is masked) or by measurement of changes in electrical resistance. However, the apparatus necessary for such determinations may not be available. Furthermore, these techniques show that a solution sometimes does not begin to freeze until well below its apparent "freezing temperature", a phenomenon known as "supercooling". It is thus generally desirable to cool a solution well below its

theoretical freezing point in order to ensure that it is completely frozen.

The rate at which freezing is carried out may also be important in preserving the activity and solubility of the material, and the most suitable rate may need to be determined experimentally. Care should be taken to avoid contamination of the material with any of the coolant used. If the volume of material in each ampoule is large, it may be necessary to increase the surface area of the contents by freezing them as a shell or as a slant.

4.2.2 *Freeze-drying*

The preferred freeze-drying equipment is the "shelf" type, in which the temperature of the material in the ampoules is reasonably uniform and is recorded continuously. If heat is applied to the shelves during the process, care should be taken to ensure that it is applied uniformly.

The use of special trays with removable bottoms results in improved thermal conductivity between the bottoms of ampoules and the tops of shelves, thus achieving greater batch uniformity. Modern freeze-driers can be sterilized by heating; if apparatus of this type is not available, partial disinfection can be achieved by wiping the shelves and walls of the drier with 70% ethanol.

It may be possible to determine the precise conditions for the successful drying of a particular substance only from previous experience with similar freeze-drying operations. Where such experience is not available, experimental runs should be carried out, but in any event it is advisable to keep the temperature of the material low, even at the cost of prolonging the time taken in freeze-drying. It is desirable for the duration of drying to be extended well beyond that found to be the minimum necessary unless a suitable device is available to show when lyophilization has been completed.

4.2.3 *Secondary drying*

Secondary drying was originally introduced because the efficiency of freeze-drying equipment was not as high as it is now and efficient stoppers (such as capillary stoppers) were not available.

Secondary drying, if necessary, may be carried out in the freeze-drier if it is suitable for this purpose, otherwise the ampoules should be transferred to desiccators containing phosphorus pentoxide. It is then essential to ensure that adequate measures are taken to prevent contamination of the material by phosphorus pentoxide powder. If lyophilized material in unsealed ampoules has been kept below room

temperature, the ampoules should, before the enclosure for secondary drying is opened, be allowed to reach the ambient temperature to prevent the condensation of moisture on to the product.

4.2.4 *Sealing*

Until the ampoules are sealed, they should be protected from light and moisture and kept at a suitable temperature, depending on the stability of the material.

It is essential to ensure that each batch of ampoules is subjected to the same conditions and, in view of the hygroscopicity of all lyophilized substances, that, before sealing, the product is exposed to atmospheric humidity for the shortest possible time. One method of achieving this is through the use of specially designed capillary plugs (*8, 9*).

After sealing, the ampoules shall be tested individually for pin-holes and cracks and defective ampoules discarded.

Ampoules may be sealed on a manifold under vacuum (1–4 Pa, or 0.01–0.03 mmHg) or after filling with a dry inert gas such as nitrogen or argon. The inert gas should be pure and completely free from oxygen, and should be dried by passing it through a desiccant. Use of a sterile gas may be appropriate in certain cases. A large batch of ampoules may have to be dealt with in portions, the size of which will depend on the number of ampoules that can be accommodated on the manifold.

"Draw" sealing is preferable to "end" sealing.

Alternatively, the ampoules may be closed with stoppers in the freeze-drier and finally sealed by fusion of the glass after removal from it, or temporarily closed outside the freeze-drier by means of specially designed stoppers that allow only very slow diffusion of gases (but do permit secondary desiccation under vacuum and expansion of the gas during sealing of the ampoule), and finally sealed by fusion of the glass.

It is usually necessary, before sealing the ampoules, to transfer them directly from the freeze-drier to desiccators, which are then evacuated.

Ampoules sealed under vacuum may be tested with a high-frequency vacuum testing coil. Sealing failures in ampoules filled with an inert gas may be detected by submerging them in methylene blue solution and exposing them to a partial vacuum for 15–20 minutes. Several minutes after having been returned to atmospheric pressure, ampoules having cracks or pin-holes will be found to contain dye.

If, because of the large number of containers to be processed, or because of the complexity of the procedure, there is a possibility that all the final containers are not homogeneous in

terms of vacuum, inert gas or residual humidity content, the different "shelves" should be properly identified so that appropriate verification is possible after sealing.

The sealed ampoules should be stored in the dark and kept at a low temperature; this is generally −20 °C, but will depend on the nature of the particular preparation.

4.3 Procedure where freeze-drying is not used

Ampoules containing liquids or solids that are not be freeze-dried should also be filled with an inert gas before sealing (see above).

This may be achieved by placing the filled ampoules in a chamber that is evacuated and filled with the gas. This process should be repeated several times to remove residual air. The inert gas should be pure and oxygen-free, and should be dried by passing it through a suitable desiccant.

When materials are to be dried in the solid state, the ampoules should be placed over phosphorus pentoxide in a desiccator, which is then evacuated to a pressure of 1–4 Pa (0.01–0.03 mmHg). After a suitable time has been allowed for drying, the ampoules may be sealed on a manifold by fusion of the glass, or after filling with a dry inert gas as described above.

4.4 Labelling

The indelible identification mark applied to the ampoules before they are filled must be sufficient to enable them to be identified during storage.

Materials intended to serve as international biological standards or reference reagents must not be labelled as such until they have been formally established by the WHO Expert Committee on Biological Standardization. Once this is done, each ampoule in the batch should be labelled to show the following items of information:

(1) The name "World Health Organization".
(2) The name of the preparation in the form "International Standard for. . .", or "International Reference Reagent of. . .". The name should be preceded by "First", "Second", "Third" etc., as applicable.
(3) The year in which the standard or reference reagent was established by the WHO Expert Committee on Biological Standardization.
(4) A code number allocated by the filling laboratory to enable the batch to be identified.

(5) The number of International Units contained in the ampoule; if applicable, the mass of solid containing one International Unit; or the number of International Units per milligram.
(6) The storage conditions necessary.
(7) The name and address of the distributing laboratory.
(8) A statement that the material is not for use in human subjects.
(9) A statement that the material should be used in accordance with the recommendations contained in the memorandum and/or instruction leaflet.

4.5 Characterization of final product

The final product should be shown, when appropriate, to be satisfactory in tests for identity, potency, content of the biological material, moisture content, residual oxygen and microbial contamination.

4.5.1 *Potency*

Potency tests are essential to confirm that the activity of the preparation is adequate for the assay or test for which it is to be used and that there has been no undue loss of potency during processing.

4.5.2 *Moisture content*

The moisture content of material in ampoules shall be determined in order to verify that drying has been adequate.

No completely satisfactory method exists for determining absolute moisture content when the moisture is present in very low concentrations in samples of biological materials. However, the available methods give results that may be used for comparative purposes. The Karl Fischer method (*10*) is frequently used. The contents of at least three ampoules should be tested separately. As the materials are usually hygroscopic, precautions must be taken to avoid erroneous results due to moisture uptake during manipulation.

Appropriate attention should be paid to cases where too low a moisture content would result in loss of potency of the material, as with certain vaccines and antibiotics.

4.5.3 *Residual oxygen*

Where appropriate, the oxygen content of the atmosphere inside the ampoule should not exceed 45 μmol/l, as measured, e.g., with an oxygen probe or a mass spectrometer.

4.6 Stability of final product

Stability tests are necessary primarily in order to estimate the length of time for which preparations used for quantitative purposes are expected to retain their potencies under storage and also to check whether the conditions under which they will be distributed to laboratories (e.g., by post) will be suitable.

When the degradation reaction of the material is a first-order reaction (i.e., of the monomolecular type), it is possible to predict how the activity will decrease with time by exposing specimens for relatively short periods (e.g., several weeks) to a range of temperatures at which denaturation will occur more rapidly than at the proposed storage temperature. From such observations, it is possible to construct an Arrhenius plot and derive a degradation constant, and thus to predict the loss of potency at the proposed storage temperature (*11–13*).

However, when degradation is not strictly a first-order reaction, the stability can be underestimated by such accelerated degradation tests. It is then necessary to conduct studies based on longer exposures of candidate reference materials at temperatures lower than those commonly used in accelerated degradation tests, the material stored at $-60\,^{\circ}C$ or lower being used to obtain the "baseline".

4.7 Records

Full records should be kept of all procedures and tests to which a material intended for use as an international standard or reference reagent has been subjected before, during and after ampouling.

5. International collaborative studies

An international collaborative study must be carried out before any candidate reference material can be considered for establishment by the WHO Expert Committee on Biological Standardization. Such studies require a considerable amount of work to be carried out by several laboratories and should be organized by a scientist familiar with the appropriate biological field and an experienced biometrician. It is important to ensure that any such international study is authorized by the body sponsoring it, and it is desirable for WHO (Biologicals) to be kept informed of plans for such studies in order

to avoid unnecessary duplication. Furthermore, when appropriate, WHO should be asked to comment on protocols for collaborative studies at an early stage in order to avoid common errors or omissions which might otherwise result in a waste of time and effort.

> The term "collaborative study" is used to denote a study carried out in an agreed way by two or more laboratories in different countries. A "collaborative assay" is a type of international study in which one standard is assayed in terms of another by previously established methods.

5.1 Aims of collaborative studies

Collaborative studies may have the following aims:

(1) To determine which of two or more candidate materials is most suitable as an international reference material. The materials may apparently be similar to one another or of clearly different types (e.g., a highly purified substance and an impure preparation).
(2) To determine whether the candidate material is suitable to serve as a standard for the assay of preparations from different manufacturers or sources.
(3) To assign a potency to the contents of the ampoules of proposed replacement international reference materials on the basis of the results of valid assays that have been statistically analysed.
(4) To determine whether different assay methods (e.g., bioassays and immunoassays) measure the same or different properties of a proposed reference material.
(5) To establish a reference material for a substance for which assay methods may not yet be of proven validity.
(6) To confirm, when necessary, that the biological material has the activity expected of it and to determine which assay method may be used in order to calibrate other similar materials.
(7) To assess the stability of a proposed international reference material by means of accelerated degradation tests.
(8) To assess, by means of appropriate tests, whether contaminating substances are present that may have biological activities different from those of the main component and/or to establish the specificity, integrity and molecular identity of the main component.

(9) To compare materials with samples from "normal populations" (e.g., to compare a standard for a coagulation factor with samples derived from pools of fresh normal plasma).

5.2 Planning

After appropriate consultation, the aims of the study should be formulated. It may be necessary to obtain and prepare samples of other test materials, in addition to the reference materials, for inclusion in the study.

Each assay should be designed to allow an assessment, based on the internal evidence, of statistical validity (i.e., for parallel-line assays, evidence of linearity and parallelism) and precision (*14*).

The number of participants will depend on the nature of the study. Generally 4–10 laboratories will be sufficient but, when very complex studies are undertaken in which new test methods are used or when assays are imprecise, it may be necessary to include a larger number of laboratories. In general, it is preferable to plan for many laboratories to carry out a few assays, rather than a few laboratories to carry out many assays.

When possible participants in the study are invited to take part in it, they should be given an outline of the aims of the study and a brief description of the materials to be included.

In a preliminary inquiry, prospective participants in collaborative studies should be asked to state:

(1) the assay methods which they could contribute to the study,
(2) the number of materials that they could conveniently compare in each assay,
(3) the number of assays that they could carry out,
(4) whether they would be willing to report their raw data using a particular reporting form as a model,
(5) whether they know how to handle the material safely.

Participants may be asked to carry out a specified minimum number of independent assays, or (for very imprecise assays) a large enough number of assays to provide a mean estimate of acceptable precision, as defined by the statistical weighting determined from all the valid assays taken together. Duplicate assays may also be requested.

For the purpose of these studies an independent assay is defined as one made using fresh dilutions from a newly opened ampoule or a fresh weighing of each material. A duplicate assay is a repeat assay using the same solutions; it does not include all the assay variables of weighing and dilution errors and is thus not truly independent.

When it is desirable for participants to use the same assay method, the procedure should be explained in detail and

sufficient time should be allowed for laboratories to become familiar with the method before carrying out the assays for the collaborative study.

5.3 Participants

The participants in collaborative studies may include national control laboratories, manufacturers of the relevant biological products, and academic or other research laboratories. Whenever possible, laboratories in several countries should be included in studies on proposed international reference materials.

Participants should be required to agree not to publish information on a proposed international reference material without the prior agreement of WHO. The premature publication of papers on a proposed international reference material can cause scientific confusion and needless concern.

Participants should be informed whether, in the report on the study, each laboratory's name will be attached to its results or whether anonymity will be preserved by the use of code numbers (the latter is the procedure generally adopted).

5.4 Materials included in collaborative studies

Biological materials included in collaborative studies must be prepared, subdivided, stored, transported and handled in such a way that all the samples of a given preparation will be identical and will not have undergone excessive degradation by the time the study has been completed. To satisfy these criteria, the careful procedures used in preparing the proposed standard itself should also be followed in handling and freeze-drying the materials.

Where feasible, samples should be coded and labelled so that participants cannot identify materials or duplicates.

In addition to candidate international reference materials, other materials studied may include:

(1) Coded duplicates for use in assessing within-laboratory reproducibility.
(2) Coded ampoules of known dilutions (e.g., 1:3 or 1:30) of the existing reference material, the candidate replacement or any other preparation included in the study. The assay results can provide evidence of the accuracy of the method used.
(3) Samples of a preparation shown to be clinically efficacious.
(4) A second or third ampoule of the bulk material from which the proposed reference material was made, in order to assess

the possibility of preparing from such ampoules further batches similar to the proposed reference material.

(5) Samples of serum or plasma containing different concentrations of the test substance, its precursor or its metabolic forms. Such samples would be useful for assessing the specificity of assays.

(6) Fresh samples of body fluids obtained locally by the participants (e.g., for the comparison of factor VIII with samples of fresh normal plasma). Guidance should be given on the selection and handling of such samples.

Participants should be informed that the coded materials are provided for the purpose of the collaborative study, and not for independent research.

5.5 Safety

Human blood, tissues and tissue extracts must be tested for hepatitis B surface antigens, antibodies to human immunodeficiency viruses, and (when appropriate tests are available) human immunodeficiency virus antigens, as well as for other relevant pathogens. In addition, participants in collaborative studies should be warned that it is extremely difficult to ensure that infectious agents are not present in biological materials, and that all suitable safety precautions should therefore be taken in handling and disposing of such materials.

If appropriate, prospective participants should be asked whether they would be prepared to accept and handle potentially harmful materials that may be contaminated with infectious agents, and to sign a form agreeing to handle and dispose of such materials on their own responsibility.

5.6 Conveyance of test materials

Test materials should be sent to participating laboratories under conditions that will ensure their stability and are in conformity with official postal or other relevant regulations governing the conveyance of such materials both nationally and internationally.

Samples sent in the frozen state must be provided with an adequate supply of ice, solid carbon dioxide or liquid nitrogen, and the containers must be insulated in a manner appropriate to the temperature conditions to be encountered during transport.

The appropriate authorities should be asked whether there are any special requirements for, or embargoes on, materials associated with special risks. Certain countries, for instance, forbid the entry of samples of viruses, while in others the

conveyance of samples contaminated with hepatitis B antigen or human immunodeficiency viruses may not be allowed.

5.7 Reporting of results

Each participating laboratory should be asked to provide information both on the assay method used, e.g., on the animals (e.g., species, strain, weight range, sex, pretreatment and method of randomization) or other test organisms employed, and on the substrates (e.g., nature, source, preparation and characterization).

Detailed records of the nature of diluent solutions and the procedure for making dilutions of test and standard materials are of particular importance in the calculation of results and the detection of causes of variation, bias or inaccuracy.

In many studies, all the essential information for each assay is entered on a common assay result sheet, which also greatly facilitates the processing of the data by computer. All results of all assays must be reported (i.e., as "raw" data). Data rejection must be avoided or explained.

Assay result sheets are designed by the study organizers in the light of the preliminary information received from the participants at the time of the initial invitation. They should be as simple as possible and their use explained.

In addition, participants should be encouraged to supply their own statistical calculations for each assay as this helps to show whether they interpret their results in the same way as the biometrician who analyses the results from all the participating laboratories.

5.8 Analysis of results

It is important to ensure that the results reported by all the participants are analysed by means of standard statistical procedures by a biometrician or someone experienced in the statistical evaluation of various types of assay.

Each assay should be analysed separately, its validity tested, and the relative potency and precision calculated (e.g., in the form of means and 95% confidence intervals). For each candidate preparation, the results of all the assays carried out by each laboratory and with each method should be combined and the potencies and confidence limits calculated.

The use of a fully comprehensive statistical procedure is recommended, and access to adequate computer facilities is therefore necessary.

Each set of results should be checked on receipt, preferably by both the scientist and the biometrician organizing the collaborative study, so that uncertainties and deficiencies can be discussed promptly with the participating laboratories.

The results of assays should be plotted as histograms because this helps to detect unusual features that may be overlooked in the study of numerical data alone.

Analysis of variance should be used to assess the significance of differences between methods and laboratories and other possible causes of variation, such as differences between the candidate reference materials included in the study. An assessment should be made of factors that may be the cause of significant heterogeneity of potency estimates, nonlinearity and differences in slopes.

5.9 Report on a collaborative study

The report on a collaborative study on a proposed international reference material, which may be submitted for publication in a scientific journal, should include the following information:

(i) Historical introduction, including the reasons for embarking on the preparation of an international reference material and a brief description of the previous reference material, if applicable.

(ii) The aims of the study and the number of laboratories taking part.

(iii) The source, nature, handling, ampouling and number of final containers of the proposed reference material, including data on the precision of fill, and the results of chemical and physical tests on the bulk and ampouled material.

(iv) The planning and design of the collaborative study and descriptions of the nature of any other materials included in it.

(v) A brief description of the statistical method used to treat the data, including any problems involved.

(vi) The total numbers of valid and invalid results, the grounds on which certain results were not included (e.g., the criteria for nonparallelism or nonlinearity were not met), the overall estimates of relative potencies by each assay method, calculated both with and without outlying results, a comparison of assay results from materials tested by different assay methods, together with their interpretation, comments

on particular factors, such as the frequency distribution and causes of differences in potency estimates, and the relative precision and bias of different assay methods.

(vii) The final figure for the overall estimate of the potency of the proposed reference material, the 95% confidence intervals and the method of deriving them, and comments on the validity of the overall estimate.

(viii) In studies on proposed first international reference materials, an assessment of the degree to which the calculation of potencies relative to the proposed reference material reduced differences between laboratories and between methods, usually expressed in the form of histograms and 95% confidence intervals.

(ix) A recommendation as to the suitability of the material to serve as a reference material together with any recommended limitations on its use (e.g., reference materials suitable for certain assay methods only), together with a recommended potency in international or other relevant units.

(x) An assessment of the stability of the material, based on accelerated degradation studies, usually expressed in the form of the predicted percentage loss of activity per year of storage at the proposed storage temperature, together with the 95% confidence intervals.

(xi) A list of the names and addresses of the participants. Unless it has been agreed to the contrary, the participants are referred to in the body of the report only by anonymous code numbers, which do not correspond to the order in which they are listed.

(xii) Tables and histograms.

(xiii) Acknowledgements, summary and references.

Each participant should be sent a copy of the draft report, accompanied by the code number of the laboratory concerned, and should be asked to state whether: (1) the laboratory's data have been correctly interpreted in the analysis; (2) the proposed material is suitable to serve as a reference material for the purpose defined; and (3) the proposed unitage is appropriate.

The final report, amended where necessary and stating that the participants have agreed on an established international reference material, should be made available to any user of it on request. It is generally used as the basis of a shorter memorandum or instruction leaflet for users that is sent out with the material.

The report should include, if applicable, the date on which the standard was established and the definition of the international unit. Any information that would be useful in current research on the subject (e.g., comparisons between assay methods) should also be included.

6. Detailed information to be provided to WHO

In addition to the information listed under section 5.9, the following information should be provided to Biologicals, WHO, in support of the submission of requests for the adoption, by the WHO Expert Committee on Biological Standardization, of candidate preparations as international reference materials.

6.1 General information

The general information required is as follows:

- The name of the substance for which an international reference material is proposed.
- The reason why a reference material is needed, and whether it is needed for the diagnosis, prevention or treatment of a specific disease. Any relevant recommendation by a scientific society or by WHO that the material should be prepared should also be included.
- The methods currently used for the assay of similar materials.
- An assessment of the possibility that similar reference materials are already (or are likely, in the near future, to be) mentioned in national pharmacopoeias and/or national requirements for the manufacture and control of biological substances, and traded in international commerce.
- A copy of the proposed instruction leaflet for users, including recommendations on the safe handling of biological reference materials, if appropriate (see the Appendix).

6.2 Pilot study

If a pilot study is performed, information should be provided on the number of final containers prepared in one successful working session, the precision of fill (the coefficient of variation, as a percentage), the vacuum achieved, the residual moisture (as a percentage), the results of accelerated degradation tests

(percentage loss of potency per year at the proposed storage temperature) and the assay method used to obtain these results.

6.3 Bulk materials

The following information on the bulk materials should be provided:

- The number of bulks from which the candidate international reference material was derived.
- The origin, nature, and approximate degree of purity of the materials, the nature of the matrix, and the nature and concentration of the stabilizers (if any).
- The composition of each final bulk (the number and volumes of all the single preparations pooled, and the volumes of diluent) and, if appropriate, the results of tests for the presence of pathogens in each individual preparation and the method used in such tests.
- The dates of the sterility tests on final bulk(s) and the result(s).
- The results of any other relevant test(s).

6.4 Filling, lyophilization, sealing and determination of stability

The information required under this heading is the following:

- The reference number of the candidate reference material.
- The address of the laboratory which filled the ampoules, lyophilized the material and sealed the ampoules.
- The dates on which these operations were performed.
- The number of ampoules used to estimate the precision of fill, the intervals at which weights were determined, and the detailed results, including the coefficient of variation (as a percentage).
- Whether secondary desiccation over phosphorous pentoxide was performed before sealing.
- The number of sealed glass ampoules in the batch.
- If sealed under vacuum, the results of testing for vacuum; otherwise, the results of tests for cracks and pin-holes.
- If not sealed under vacuum, the gas under which the material was sealed, its purity, the method used for determining the residual oxygen content of the ampoules and the results of such determinations.
- The number of ampoules tested for residual moisture, the method used and the results obtained (as a percentage of the dry weight).

- The date of the test for sterility, the number of final containers tested and the results.
- The laboratories that obtained the stability data and the assay method(s) used to obtain them.
- The number of containers exposed, the duration of the exposure and the temperatures, and the activity remaining in each container after exposure, together with the 95% confidence intervals.
- The predicted loss of activity, expressed as a percentage per year, at −20 °C, +4 °C and +20 °C and, if applicable, at the appropriate storage temperature.
- The address of the place of storage (if different from that of fill) and the name of the present custodian.
- The actual temperature of storage.
- The number of ampoules offered to WHO after visual inspection has shown them to be satisfactory.

6.5 Results of the collaborative study

The following information on the results of the collaborative study is required:

- The assay methods used and the laboratories which used them.
- For each assay method, the number of assays that each laboratory was asked to perform and the number actually carried out.
- The way in which the linearity and parallelism of the dose–response curves were established.
- For each (coded) laboratory using a given assay method overall, the within-assay variation and the overall between-assay variation.

7. Establishment of an international biological standard or reference reagent

A preparation can be established as an international reference material by the WHO Expert Committee on Biological Standardization when: (1) the report on a collaborative study on it has been approved by all the participants; (2) it is agreed by the participants that the preparation should be proposed as an international standard or reference reagent; and (3) all queries raised

by members of the Expert Advisory Panel on Biological Standardization after they have examined the information listed under sections 5.9 and 6.1–6.5 have been answered satisfactorily. It is then endorsed by the Director-General of WHO. Every few years, changes in the WHO list of international biological standards and reference reagents are proposed for adoption by the World Health Assembly, such changes being adopted in the form of resolutions containing certain recommendations to Member States (*15*). In addition, a list of international standards and reference reagents (*6*) is published every few years. Any preparations that have been established or discontinued as reference materials since the list was last published are included in an appendix to the report of the most recent meeting of the WHO Expert Committee on Biological Standardization. Such appendices should be consulted until such time as a revised version of the complete list is produced. In addition, custodian laboratories maintain up-to-date catalogues which are available on request to potential users.

PART B. GUIDELINES FOR THE PREPARATION, CHARACTERIZATION AND CALIBRATION OF NATIONAL OR LABORATORY WORKING STANDARDS AND REFERENCE REAGENTS FOR BIOLOGICAL SUBSTANCES

1. Introduction

International biological reference materials are distributed free of charge to national control authorities for the purpose of calibrating national reference materials so that the activities of such preparations can be expressed in international or other relevant units. A manufacturer undertaking the assay of a large number of batches of a biological substance is usually expected to establish a laboratory working reference material in order to avoid making excessive demands on the supplies of the national standard. The activities of such laboratory preparations should be calibrated in International Units by comparison with the national reference material or, where this is not possible, by direct comparison with the international reference material.

The definitions of national and laboratory working standards and reference reagents for biological substances are similar to those of

their international counterparts (see Part A, section 1), except that they are intended for national use only.

Where an international reference material does not exist, a national control laboratory may need to establish a national reference material independently of WHO and, if appropriate, define a unit of activity.

Whenever possible, countries in a given region should jointly prepare regional reference materials (RRM) which can also be used as national reference materials. By so doing, countries are likely to produce and use reference materials that are better characterized and of a higher quality than would otherwise be the case. RRMs are currently being prepared by certain countries adhering to the *European pharmacopoeia.*

2. Assessment of need and procurement of material

A national standard may be needed for the following purposes:

(1) The assessment of a biological product used in medical practice and being considered for licensing in the country concerned, regardless of whether the product is manufactured locally or imported.
(2) The performance of clinical laboratory tests that require reference materials having defined activities.
(3) The comparison of sets of research data on materials used in treatment and diagnosis that require assay.

Most of the technical considerations that apply to bulks used in the preparation of international reference materials also apply to those used for national standards (see Part A, section 2.2). The purpose for which a material is required should be explained to the candidate supplier, who is most often the manufacturer. Homogeneity is the most important requirement. The composition of a national standard should be typical of that of the materials to be assayed against it.

In some instances, bulk materials will be obtained for distribution into containers by the national control laboratory; in other cases, material may be obtained in the form of final containers, which may either be glass-sealed or, exceptionally, closed with rubber caps.

3. Distribution into final containers

In the preparation of national and laboratory reference materials, homogeneity is essential and all the considerations and precautions

set out in Part A, section 3 should be taken into account, but certain aspects of the procedures may sometimes be simplified.

Provided that the material is of adequate stability (see Part B, section 5), stoppered vials are acceptable.

4. Processing of filled containers

With the exception of those given in Part A, section 4.4 (labelling), most of the recommendations included in Part A are also applicable to national or laboratory working reference materials.

5. Calibration of national reference materials

The calibration of national reference materials should be organized by the relevant national control laboratory or other national body, or by a laboratory acting on behalf of the national control authority.

A national standard is generally calibrated in terms of International Units by comparison with an international reference material.

The principles on which calibration is based are similar to those listed in Part A, sections 3–5. However, the materials are less fully characterized and collaborative studies are usually less comprehensive, e.g., such studies do not have to involve laboratories from more than one country, especially since the responsibilities involved in establishing national reference materials for biological substances are different from those for international reference materials. In some instances, as few as two participating laboratories may be sufficient. Great care should be taken to calibrate national reference materials or individual reagents as accurately as possible in order to avoid systematic overestimation or underestimation of potency. Accuracy can be improved by performing a number of replicate assays. Reports on assays by participants need not be as detailed as those considered in Part A, section 5.7, but should be based on the same principles.

Copies of final reports should be retained by national control authorities and summaries made available to users of national standards.

Unless it has been established that the materials are stable, the activities of national reference materials should be compared regularly with those of the relevant international standards.

If there is any indication of a loss of activity, steps should be taken to replace the materials. This also applies to laboratory standards, except that the materials used as references are the national standards.

AUTHORS

The authors of the first Guidelines for the Preparation and Establishment of Reference Materials and Reference Reagents for Biological Substances are listed in WHO Technical Report Series, No. 626, 1978, p. 137. A first draft of these revised Guidelines was prepared by Dr P. Sizaret, Scientist, and Dr D. Magrath, Chief, Biologicals, WHO, Geneva, Switzerland. A second draft was prepared by Dr S.L. Jeffcoate, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr P. Sizaret and Dr D. Magrath.

ACKNOWLEDGEMENTS

Acknowledgement is made to the following experts for their comments and advice: Dr M. Hilleman, Director, Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, Westpoint, PA, USA; Mr J. Lyng, State Serum Institute, Copenhagen, Denmark; Dr R. Netter, Director-General, National Health Laboratory, Ministry of Solidarity, Health, and Social Protection, Paris, France; Dr J. Petricciani, Vice-President for Medical and Regulatory Affairs, Pharmaceutical Manufacturers Association, Washington, DC, USA; Dr P. Rousseau, Armand Frappier Institute, Laval, Quebec, Canada; Dr W.W. Wright, The United States Pharmacopeia, The National Formulary, Rockville, MD, USA; Dr Zhou Haijun, Director, National Institute for the Control of Pharmaceutical and Biological Products, Temple of Heaven, Beijing, China.

REFERENCES

1. WHO Technical Report Series, No. 626, 1978.
2. WHO Technical Report Series, No. 700, 1984.
3. WHO Technical Report Series, No. 745, 1987.
4. WHO Technical Report Series, No. 760, 1987.
5. WHO Technical Report Series, No. 771, 1988.
6. *Biological substances. International standards and reference reagents, 1986.* Geneva, World Health Organization, 1987.
7. WHO Technical Report Series, No. 594, 1976.
8. CAMPBELL, P.J. International biological standards and reference preparations. I. Preparation and presentation of materials to serve as standards and reference preparations. *Journal of biological standardization,* **2**: 249–258 (1974).
9. CAMPBELL, P.J. International biological standards and reference preparations. II. Procedures used for the production of biological standards and reference preparations. *Journal of biological standardization,* **2**: 259–267 (1974).

10. *The International Pharmacopoeia*. 3rd edition. *Volume 1: general methods of analysis*. Geneva, World Health Organization, 1979, p. 134.
11. KIRKWOOD, T.B.L. Predicting the stability of biological standards and products. *Biometrics*, **33**: 736–742 (1977).
12. KIRKWOOD, T.B.L. & TYDEMAN, M.S. Design and analysis of accelerated degradation tests for the stability of biological standards II. A flexible computer program for data analysis. *Journal of biological standardization*, **12**: 207–214 (1984).
13. KIRKWOOD, T.B.L. Design and analysis of accelerated degradation tests for the stability of biological standards III. Principles of design. *Journal of biological standardization*, **12**: 215–224 (1984).
14. KIRKWOOD, T.B.L. ET AL. Statistical aspects of the planning and analysis of collaborative studies on biological standards. *Journal of biological standardization*, **14**: 273–287 (1986).
15. SIZARET, P. The preparation of international reference materials for biological substances. *Journal of biological standardization*, **16**: 129–137 (1988).

Appendix

Example of a statement on safe handling which may need to be included in instruction leaflets for users of international or other biological reference materials

Many international and national biological standards and reference reagents are derived from human or animal tissues that might harbour potentially harmful infectious agents. The bulk source materials from which the preparations are obtained have, where relevant, been screened for evidence of markers of contamination by hepatitis B virus and human immunodeficiency viruses. Furthermore, many reference materials have been purified and/or have undergone other procedures, such as heat treatment, that have been shown, or are expected, to remove or inactivate such microbial agents.

Nevertheless, all the common-sense safety measures recommended for the laboratory handling of liquid or freeze-dried samples of human blood should be adopted for the handling and disposal of biological reference materials and their containers.[1]

It is important to ensure that, in the handling of reference materials of certain microorganisms, microbiological procedures appropriate to the organism concerned are followed.

[1] *Laboratory biosafety manual.* Geneva, World Health Organization, 1983.

World Health Organization. Technical Report Series, No. 800, 1990

Annex 5

BIOLOGICAL SUBSTANCES: INTERNATIONAL STANDARDS AND REFERENCE REAGENTS

A list of international biological standards, international biological reference preparations, and international biological reference reagents is issued as a separate publication.[1] Copies may be obtained from appointed sales agents for WHO publications or from: Distribution and Sales, World Health Organization, 1211 Geneva 27, Switzerland.

The Expert Committee made the following changes to the previous list.

Additions

Antibiotics		
Netilmicin	4810 IU/ampoule	First International Standard 1989
Blood products		
High molecular weight urokinase	4300 IU/ampoule	First International Standard 1989
Streptokinase	700 IU/ampoule	Second International Standard 1989
Blood coagulation factor VIII:C concentrate, human	6.3 IU/ampoule	Fourth International Standard 1989
Endocrinological and related substances		
Salmon calcitonin	128 IU/ampoule	Second International Standard 1989

[1] *Biological substances: international standards and reference reagents, 1986.* Geneva, World Health Organization, 1987.

Eel calcitonin	88 IU/ampoule	First International Standard 1989

Cytokines

Interleukin-1 alpha	117 000 IU/ampoule	First International Standard 1989
Interleukin-1 beta	100 000 IU/ampoule	First International Standard 1989

(These substances are held and distributed by the National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.)

Discontinued

Antibiotics

Clindamycin	837 IU/mg	First International Reference Preparation 1971
Lincomycin	881 IU/mg	First International Reference Preparation 1965
Cefalotin	938 IU/mg	First International Reference Preparation 1965
Procaine benzylpenicillin in oil with aluminium monostearate		Second International Reference Preparation 1965

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Annex 6

REQUIREMENTS FOR BIOLOGICAL SUBSTANCES AND OTHER DOCUMENTS

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 are formulated by international groups of experts and are published in the Technical Report Series of the World Health Organization,[1] as listed here.

I. Requirements

1. General Requirements for Manufacturing Establishments and Control Laboratories
   Revised 1965, WHO TRS **323** (1966)
2. Requirements for Poliomyelitis Vaccine (Inactivated)
   Revised 1981, WHO TRS **673** (1982)
   Addendum 1985, WHO TRS **745** (1987)
3. Requirements for Yellow Fever Vaccine
   Revised 1975, WHO TRS **594** (1976)
   Addendum 1987, WHO TRS **771** (1988)
4. Requirements for Cholera Vaccine
   Revised 1968, WHO TRS **413** (1969)
   Addendum 1973, WHO TRS **530** (1973)
5. Requirements for Smallpox Vaccine
   Adopted 1966, WHO TRS **323** (1966)
6. General Requirements for the Sterility of Biological Substances
   Revised 1973, WHO TRS **530** (1973)

[1] Abbreviated here as WHO TRS.

7. Requirements for Poliomyelitis Vaccine, Oral
   Revised 1989, WHO TRS **800** (1990)
8 & 10. Requirements for Diphtheria, Tetanus, Pertussis and Combined Vaccines
   Revised 1989, WHO TRS **800** (1990)
9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate
   Revised 1966, WHO TRS **361** (1967), **discontinued**
11. Requirements for Dried BCG Vaccine
   Revised 1985, WHO TRS **745** (1987)
   Amendment 1987, WHO TRS **771** (1988)
12. Requirements for Measles Vaccine (Live)
   Revised 1987, WHO TRS **771** (1988)
13. Requirements for Anthrax Spore Vaccine (Live, for Veterinary Use)
   Adopted 1966, WHO TRS **361** (1967)
14. Requirements for Human Immunoglobulin
   Adopted 1966, WHO TRS **361** (1967), *replaced by Requirements No. 27*
15. Requirements for Typhoid Vaccine
   Adopted 1966, WHO TRS **361** (1967)
16. Requirements for Tuberculins
   Revised 1985, WHO TRS **745** (1987)
17. Requirements for Influenza Vaccine (Inactivated)
   Revised 1978, WHO TRS **638** (1979)
18. Requirements for Immune Sera of Animal Origin
   Adopted 1968, WHO TRS **413** (1969)
19. Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)
   Adopted 1969, WHO TRS **444** (1970)
20. Requirements for *Brucella abortus* Strain 19 Vaccine (Live, for Veterinary Use)
   Adopted 1969, WHO TRS **444** (1970)
   Addendum 1975, WHO TRS **594** (1976)
21. Requirements for Snake Antivenins
   Adopted 1970, WHO TRS **463** (1971)
22. Requirements for Rabies Vaccine for Human Use
   Revised 1980, WHO TRS **658** (1981)
23. Requirements for Meningococcal Polysaccharide Vaccine
   Adopted 1975, WHO TRS **594** (1976)
   Addendum 1976, WHO TRS **610** (1977)

Addendum 1977, WHO TRS **626** (1978)
Addendum 1980, WHO TRS **658** (1981)

24. Requirements for Rubella Vaccine (Live)
Adopted 1976, WHO TRS **610** (1977)
Addendum 1980, WHO TRS **658** (1981)
25. Requirements for *Brucella melitensis* Strain Rev. 1 Vaccine (Live, for Veterinary Use)
Adopted 1976, WHO TRS **610** (1977)
26. Requirements for Antimicrobic Susceptibility Tests
I. Agar diffusion tests using antimicrobic susceptibility discs
Revised 1981, WHO TRS **673** (1982)
Addendum 1982, WHO TRS **687** (1983)
Addendum 1985, WHO TRS **745** (1987)
Addendum 1987, WHO TRS **771** (1988)
Addendum 1989, WHO TRS **800** (1990)
27. Requirements for the Collection, Processing, and Quality Control of Blood, Blood Components, and Plasma Derivatives
Revised 1988, WHO TRS **786** (1989)
28. Requirements for Influenza Vaccine (Live)
Adopted 1978, WHO TRS **638** (1979)
29. Requirements for Rabies Vaccine for Veterinary Use
Adopted 1980, WHO TRS **658** (1981)
30. Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy
Revised 1982, WHO TRS **687** (1983)
31. Requirements for Hepatitis B Vaccine prepared from Plasma
Revised 1987, WHO TRS **771** (1988)
32. Requirements for Rift Valley Fever Vaccine
Adopted 1981, WHO TRS **673** (1982)
33. Requirements for Louse-Borne Human Typhus Vaccine (Live)
Adopted 1982, WHO TRS **687** (1983)
34. Requirements for Typhoid Vaccine (Live Attenuated, Ty 21a, Oral)
Adopted 1983, WHO TRS **700** (1984)
35. Requirements for Rift Valley Fever Vaccine (Live, Attenuated) for Veterinary Use
Adopted 1983, WHO TRS **700** (1984)
36. Requirements for Varicella Vaccine (Live)
Adopted 1984, WHO TRS **725** (1985)

37. Requirements for Continuous Cell Lines used for Biologicals Production
    Adopted 1985, WHO TRS **745** (1987)
38. Requirements for Mumps Vaccine (Live)
    Adopted 1986, WHO TRS **760** (1987)
39. Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques in Yeast
    Adopted 1986, WHO TRS **760** (1987), *replaced by Requirements No. 45*
40. Requirements for Rabies Vaccine (Inactivated) for Human Use Produced in Continuous Cell Lines
    Adopted 1986, WHO TRS **760** (1987)
41. Requirements for Human Interferons Made by Recombinant DNA Techniques
    Adopted 1987, WHO TRS **771** (1988)
42. Requirements for Human Interferons Prepared from Lymphoblastoid Cells
    Adopted 1988, WHO TRS **786** (1989)
43. Requirements for Japanese Encephalitis Vaccine (Inactivated) for Human Use
    Adopted 1987, WHO TRS **771** (1988)
45. Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques
    Adopted 1988, WHO TRS **786** (1989)

Requirements for Immunoassay Kits (unnumbered)
Adopted 1980, WHO TRS **658** (1981)

II. Other documents

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)
WHO TRS **565** (1975)

Development of national assay services for hormones and other substances in community health care
WHO TRS **565** (1975)

Report of a WHO Working Group on the Standardization of Human Blood Products and Related Substances
WHO TRS **610** (1977)

Guidelines for quality assessment of antitumour antibiotics
WHO TRS **658** (1981)

The national control of vaccines and sera
WHO TRS **658** (1981)

Procedure for approval by WHO of yellow fever vaccines in connection with the issue of international vaccination certificates
WHO TRS **658** (1981)

A review of tests on virus vaccines
WHO TRS **673** (1982)

Standardization of interferons (reports of WHO informal consultations)
WHO TRS **687** (1983)
WHO TRS **725** (1985)
WHO TRS **771** (1988)

Report of a WHO Meeting on Hepatitis B Vaccines Produced by Recombinant DNA Techniques
WHO TRS **760** (1987)

Laboratories approved by WHO for the production of yellow fever vaccines, revised 1987
WHO TRS **771** (1988)

Procedure for evaluating the acceptability in principle of vaccines proposed to United Nations agencies for use in immunization programmes, revised 1988
WHO TRS **786** (1989)

Guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances
WHO TRS **800** (1990)

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