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 ($P \leq 0.05$), 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 ± 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).

The second draft of these Requirements was drawn up in June 1988 by the following participants at an informal WHO consultation:

Dr E. Griffiths, Department of Bacteriology, National Institute for Biological Standards and Control, Potters Bar, Herts., England

Dr C. Hardegree, Director, Division of Bacterial Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA

Dr P. Knight, Quality Assurance, Wellcome Biotech, Beckenham, Kent, England (*Rapporteur*)

Dr J.G. Kreeftenberg, Head, Laboratory for Control of Bacterial Vaccines, National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands

Mr J. Lyng, Department of Biological Standardization, State Serum Institute, Copenhagen, Denmark

Dr C. Manclark, Chief, Laboratory of Pertussis, Division of Bacterial Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA

Dr T. Matuhasi, Consulting Research Scientist, Okinaka Memorial Institute for Medical Research, Tokyo, Japan

Dr G. Nyerges, Department of Control of Vaccines and Sera, National Institute of Hygiene, Budapest, Hungary

Dr S.N. Saxena, Director, Central Research Institute, Kasauli, India

Dr Yu Yong Pin, National Institute for Control of Pharmaceutical and Biological Products, Temple of Heaven, Beijing, China

WHO Secretariat

Dr V. Grachev, Scientist, Biologicals, World Health Organization, Geneva, Switzerland

Dr D. Magrath, Chief, Biologicals, World Health Organization, Geneva, Switzerland

Dr J. Milstien, Scientist, Biologicals, World Health Organization, Geneva, Switzerland

Dr P. Sizaret, Scientist, Biologicals, World Health Organization, Geneva, Switzerland (*Secretary*)

The third draft of these Requirements was prepared in 1989 by Dr P. Sizaret, and Dr D. Magrath, Chief, Biologicals, World Health Organization, Geneva, Switzerland.

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.

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[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.

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[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.

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[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.