BLG/UNDP/77.2 Rev.1

WHO

MANUAL FOR THE PRODUCTION AND CONTROL OF VACCINES

TETANUS TOXOID

WORLD HEALTH ORGANIZATION

ORGANISATION MONDIALE DE LA SANTÉ

BLG/UNDP/77.2 Rev.1

ENGLISH ONLY

MANUAL FOR THE PRODUCTION AND CONTROL OF VACCINES

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FOREWORD

A number of countries are wishing to produce vaccines at the national level for which they require the necessary technical information. With this aim the present manual[1] has been prepared.

It should be stressed that this manual is intended only to provide general information on methods - preferably the least complicated ones - for the production of a tetanus vaccine of acceptable quality meeting the WHO Requirements[2] and to describe the necessary tests. There exist other production methods, which may lead to a similar result. The manual, therefore, does not necessarily express any preference for the methods chosen and should not be regarded as a description of those production methods preferred by WHO. This is true also for all (bio)chemical preparations, specified in the manual, as well as for the manufacturers and special equipment mentioned.

If clarification is required on any point, reference should be made to the WHO Secretariat (Chief, Biologicals).

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[1] The manual was prepared by the following Consultants and members of the WHO Secretariat:

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[2] WHO Technical Report Series 1979 (to be be published); Appendix T.27 of this manual.

CONTENTS

TETANUS TOXOID

(Requirements for Biological Substances No. 10 (revised 1978)) See Appendix T.27

T1. INTRODUCTION

T1.1 Description of the vaccine (Part A, Section 1.2, page 41) See Appendix T.27

Tetanus toxoid is prepared from the toxin produced by the growth of a highly toxigenic strain of Clostridium tetani in a suitable medium. The supernatant fluid, which contains the toxin, is separated from the organisms; the toxin so separated from the organisms is detoxified and purified.

T1.2 Efficacy of the vaccine

Tetanus toxoid is the immunizing antigen protecting against the adverse effects of infection by tetanus organisms. Although the toxoid induces the production of tetanus antitoxin and will thereby protect against the lethal effects of tetanus toxin, it does not necessarily prevent the growth of the organisms.

The toxoid is a very good antigen and even plain toxoid (a toxoid without the addition of an adjuvant) given in adequate dosage can be relied upon to protect when used for primary immunization. It is common, however, for the toxoid to be adsorbed on to an adjuvant (usually aluminium hydroxide or phosphate). Tetanus toxoid, either plain or adsorbed, is rarely given alone in children for whom it is much more commonly included in a combined vaccine together with diphtheria toxoid and pertussis vaccine.

Tetanus toxoid is used alone in pregnant women in order to prevent neonatal tetanus (tetanus neonatorum). It is given also to casualties and is used prophylactically in groups in which tetanus is an occupational hazard, e.g. those working with machines.

A tetanus toxoid complying with the WHO Requirements and given in three doses, suitably spaced for primary immunization, will protect against tetanus; a reinforcing dose should be given at school entry (about five years of age).

T1.3 Suggested method of production

This manual describing the production and control of tetanus toxoid suggests methods of production that have been shown to give a satisfactory product. The method will not necessarily utilize the most refined, expensive and modern equipment that may be in use in some laboratories having many years' experience in the production of the toxoid, but the suggested method is known to yield toxoid of consistently good quality. It is only after a new production area has had a number of years of experience in the production of the toxoid by a relatively simple method that more refined methods should be considered.

The controls applied both during production and on the final product follow closely those included in the WHO Requirements (see Appendix T.27). A most important factor in vaccine manufacture is consistency of production of quality vaccine and additional controls to ensure this are suggested.

In attempting to make the manual as widely applicable as possible, it has been necessary in some instances to suggest alternative control tests. If a laboratory has no experience of any of the suggested tests the one of choice should be the one most easily carried out having regard to the availability of materials and resources.

In general the methods will comply with the requirements of most national authorities for the production and control of biological substances. If there is a divergence in any particular country, permission to use methods other than those recommended must be obtained from the national control authority.

T1.4 WHO Requirements (Technical Report Series (1964), 293)
See Appendix T.27

The WHO Requirements for Tetanus Toxoid were first formulated in 1964; since then there have been several developments in vaccine production and the Requirements were therefore revised in 1978. The control procedures suggested in this manual follow them closely. Although the method of potency control suggested in the manual may not be identical with that used by many countries, the quality of the final toxoid will satisfy the requirements of all countries.

T1.5 Glossary of terms

Kf	=	Flocculation time (in minutes) as observed in the flocculation reaction.
Lf	=	Limes flocculationis; the amount of toxin or toxoid which when mixed with 1 International Unit of antitoxin gives a Ramon flocculation in the shortest time.
Lf/mg N	=	Flocculation units per milligram of total nitrogen as determined by the Kjeldahl analysis.
Lf/ml PN	=	Flocculation units per milligram of protein nitrogen (PN) which is usually determined by the precipitation of the proteins with trichloroacetic acid.
I.U.	=	International Unit is the specific activity of a stated amount of the International Standard as defined by the WHO Expert Committee on Biological Standardization.

L+ = The minimum amount of toxin which when combined with 1 I.U. of antitoxin kills an animal of defined weight in four days (the L+ is dependent upon the animal species used).

L+/10 = The minimum amount of toxin which when combined with 0.1 I.U. of antitoxin kills a mouse of a defined weight in four days. (For tetanus)

Lr = The minimum amount of toxin which when combined with a fixed amount of antitoxin (usually 0.002 I.U. of antitoxin) in the volume of 0.2 ml causes a local skin reaction that is just visible. (Only for diphtheria)

Ld_{50} = The amount of toxin that kills 50% of a group of animals within four days (the LD_{50} differs for different animal species).

M.L.D. = Minimal lethal dose, the amount of toxin which kills animals within four days (M.L.D. is different for different animal species). In general the M.L.D. has been replaced by the LD_{50}.

A.B.V. = Antitoxin binding value, a value which defines the toxin plus toxoid in a mixture (determined in animals).

O.U. = Opacity Unit is the measure of opacity as determined in comparison with the International Reference Preparation of Opacity.

T.C.P. = Total combining power, the reciprocal of the volume of toxoid which when diluted is found to be equivalent to 1 International Unit.

ED_{50} = The dose of a vaccine which protects 50% of the immunized animals against a challenge dose of virulent bacteria or toxin.

Single harvest. The toxic filtrate 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 toxoid present in a single container from which the final containers are filled either directly or through one or more intermediate containers.

Final lot or filling lot, A collection of sealed final containers that is homogenous with respect to the risk of contamination during filling. A final lot must, therefore, be filled in one working session.

T2. PREMISES

(Part A, Section, page 13) See Appendix T.29
For more details see also: Manual for the Design, Equipping and Staffing of Facilities for Production and Quality Control of Bacterial Vaccines (BLG/UNDP/78.1)

T2.1 Design of premises

Tetanus toxin must be produced in separate premises because Clostridium tetani is a spore bearing organism. The most important requirements for the premises in which toxin is to be produced are that they should be clean, comfortable to work in, have control of temperature and humidity, include all the

essential facilities, and have built-in safety measures against hazards such as contamination. The lighting should be such that all equipment can be seen and operated without difficulty. It is advisable that the laboratories should be supplied with filtered air or that work should be done in laminar-flow cabinets that are monitored regularly to ensure that they are operating correctly. It should be stressed, however, that the use of laminar-flow cabinets does not obviate the need for a high standard of microbiological technique.

T2.2 Quality of materials

The main objective in the choice of building materials for a laboratory is that the shedding or collection of dust shall be avoided. This applies to all surfaces including the floor, which should be covered with an impervious epoxy resin surface. Where there is heavy traffic, quarry tiles or terrasso floors are to be preferred. The walls and ceiling should be of a high quality hard plaster sealed by painting with a washable material that does not flake. Ledges, that inevitably collect dust, should be avoided and benches should be impervious and without cracks or crevices. Suitable construction materials for benches include laminated plastics, metal and wood covered with an epoxy resin. They should be able to withstand frequent swabbing and disinfection without deterioration. It must be possible to spray the whole area with a disinfectant without damage to any of the materials.

T2.3 Changing facilities

Modern practices in the production of virus vaccines made from attenuated strains of living organisms demand that, for entry into a production area, all outdoor clothing be removed, and that the operators be cocooned in sterile clothing. This is because there are no purification or inactivation steps involved in the production process. Although such stringent precautions are less important in the production of a toxoid or bacterial vaccine in which the organisms are killed by a chemical or in which purification is involved, it

is essential to have some barrier between the outside dusty contaminated atmosphere and the production area. The areas set aside for the culture of organisms, processing, blending and filling of final vaccine must be approachable only through an anteroom equipped for washing, change of footwear, and the donning of protective clothing such as clean gowns that do not shed dust or fibres. Where open operations are carried out, either a cap and mask or a total head hood should be worn. Access to this anteroom must be from other parts of the production area and not directly from the exterior.

A production unit and its staff can be used for the production of more than one vaccine in a sequential production programme. The only vaccine requiring a separate facility is tetanus.

T2.4 Premises for breeding and keeping animals

It is a commonly mistaken view that animals can be kept anywhere and under any conditions. If reliance is to be placed upon the results of animal experiments, the animals must be housed under conditions in which they will remain healthy and will not be subjected to cross infection.

They require:

(a) clean living conditions with plenty of light and good ventilation. Animals generate a great deal of body heat as well as humidity and overcrowding can be disastrous.

(b) a good balanced diet with some variety. It is important that contamination of food and bedding, which usually is caused by wild rodents, should be prevented;

(c) living quarters that can be cleaned daily and disinfected between batches of animals.

It is important to control the bacterial, fungal and helminth population in the breeding stock. Many intercurrent infections are able to affect the results of animal experiments. Such infections should be kept to a minimum by segregation of animals from different sources, selective breeding from healthy stock and as a last resort by suitable treatment with therapeutic agents.

The quality control of tetanus toxoid requires a large supply of healthy animals[1], and such control should not be attempted unless good animals are available either from one's own facility or from a regular supplier.

Such an animal facility should be capable of housing and if necessary breeding the animals used for tests on a number of vaccines such as diphtheria, pertussis and tetanus, and may be used also for the testing of cholera and typhoid vaccines. For the testing of these vaccines only mice and guinea-pigs are needed in appreciable numbers.

T3. EQUIPMENT FOR TOXIN PRODUCTION

(Part A, Section 2, page 42) See Appendix T.27

T3.1 Equipment for production in static culture

The equipment used for the production of toxin will depend upon the system chosen for cultivation of the bacteria, but in principle no expensive equipment is needed. It can be carried out in wide-mouthed bottles, tall beakers or, for larger scale production, in stainless-steel vessels.

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[1] The UFAW Handbook on the Care and Management of Animals (Ed. Churchill Livingstone, Edinburgh, London, New York, 5th Edition, 1976) is a most useful reference for all aspects involved in the care of animals.

Convenient sizes of containers range from 5-litre beakers containing 3.5 litres of medium to 20-litre stainless-steel containers containing 15 litres of medium. The beakers are covered with a thin layer of non-absorbent cotton-wool 1-2 cm thick held between two layers of hydrophil gauze. An injection needle or a glass tube of about 15 mm diameter, open at both ends, is passed through the thick covering and held in position by rubber washers which fit snugly to the needle or tube on both sides of the cotton cover. The upper end of the needle or glass tube is covered either with an aluminium foil or plugged with cotton.

Mueller & Miller used Erlenmeyer flasks of 2 litres capacity containing 1700 ml of medium.

T3.2 Fermenter production

For large-scale production stainless-steel fermenters can be used of any size from 20 litres upwards. They need not be highly instrumented since the growth of tetanus requires only a control on impeller speed, air supply and temperature, and the fermenters may be purchased from commercial manufacturers or made to order by a good fabricator of stainless-steel. Although the initial capital investment may seem high they have an assured working life of at least 10 years and are likely to present the minimum in demands on servicing if carefully designed.

The equipment used for the production of tetanus toxin must be kept separate at all times from all other equipment.

T4. STAFF

T4.1 Qualifications and numbers

The number of staff involved in the production of tetanus toxoid depends to some extent upon the volume of toxoid to be produced and the number of batches required.

It is not advisable to employ staff who have no knowledge of microbiology but a well-trained nucleus of staff can train other staff quite quickly. The production of the media would require one graduate scientist and one technician.

Maintenance of seed culture, preparation of inoculum, growth of organisms, separation, purification and detoxification of toxin, can be carried out by a graduate microbiologist with some years' experience and three technicians.

Quality control of the bulk toxoid and the final product will require also a graduate scientist and one technician.

The animal facility should be supervised by a senior technician with adequate experience and training in the maintenance of small animals. Provision should be made for professional veterinary consultation when required.

Finally, it is important that in every laboratory for vaccine production at least one mechanical engineer should be available to be responsible for the equipment.

T4.2 Experience

The technical staff should have had an education beyond normal schooling. In further education they should have had an opportunity of studying microbiology and have a working knowledge of sterile techniques and the handling of bacteria. The scientific staff should have spent at least three to six months in a production unit actively engaged in the manufacture and control of bacterial vaccines.

T4.3 Health

All staff should be in good health and should be immunized against or known to have natural immunity against any infectious organisms that they may be required to handle. They should be immunized against tuberculosis and not be suffering from any other respiratory or diarrhoeal disease. Any staff members having a septic wound should not enter the production area.

The technical staff looking after the breeding colony or experimental animals should also be in good health and free from any latent infection.

T4.4 Organization of activity

Although the same staff can be used also for the production of all vaccines they must not move from the tetanus toxin production area into the other production areas on the same working day. The staff may go into the tetanus area, however, after working in the other areas.

Quality control and production should be administratively separate but it is important that quality control staff have first-hand knowledge and experience of production. There is no need for animal testing to be duplicated, but it is important that the detailed results of all "in process" and final tests should be available to both production and quality control staff.

In any event, accurate and clear permanent records signed legibly and dated must be kept, and must be made available at all times to the production and quality control staff as well as to the appropriate inspectors from local health authorities. Examples of forms suitable for keeping such records are shown in Appendices T.25 and T.26. Though the production facilities for tetanus toxin cannot be used for the manufacture of other bacterial vaccines, the staff should be trained in the production of a number of other bacterial vaccines as it can be used for this purpose in other areas at times when tetanus production is not in progress. Similarly, the quality control staff should be capable of the control of all bacterial vaccines.

T5. MEDIA

The medium most commonly used for the cultivation of Cl. tetani is the medium originally described by Mueller & Miller (J. Immunol. (1947) 56, 143).

Some modifications in the formulation of the original medium have been made and these are also described in Appendix T.1.

The main constituent of the medium is a tryptic digest of casein. This may be prepared in the laboratory or bought commercially.

T6. STRAINS OF CL. TETANI

T6.1 Obtaining the strain

In the production of tetanus toxin, the strain plays an important role. The strain which has been most commonly used in combination with the media described originates from the New York State Department of Health and should be obtained from a laboratory which is experienced in toxin production.[1] The strain is a highly toxigenic strain and differs considerably from normal Cl. tetani strains.

Although all tetanus toxoids are made from a few highly toxigenic strains, it is not possible to obtain the same yields of toxin by a strain if the growth medium is markedly changed in its composition. A new production laboratory, therefore, will need to obtain a strain and undergo a period of adaptation of the organism to the medium selected for growth before maximum yields may be expected.

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[1] New manufacturers may obtain data concerning a suitable strain from the WHO Secretariat (Chief, Biologicals).

T6.2 Maintenance of the strain

The stock cultures prepared from the original strain have to be maintained in the freeze-dried form to preserve viability. The working seed culture prepared from this can be maintained by daily subculture in glucose broth until the culture shows a fall in toxin production. The daily subcultures are grown in an anaerobic jar.

T6.3 Preparation for production

The containers of the culture medium that have been autoclaved and rapidly cooled in running tapwater or in ice cold water are transferred to the inoculation room where the 24-hour-old seed cultures and the required number of sterile syringes or Pasteur pipettes are kept. The purity of each seed culture is checked by the examination of Gram stained smears and only those cultures found to be pure are used for the inoculation of the freshly prepared and cooled medium.

T7. PRODUCTION OF VACCINE

In order to follow more easily the production of purified tetanus toxoid suggested flow charts are shown in Appendix T.26. These indicate at each step in the production where materials are added as well as where samples are taken for testing. The tests applied are also specified.

T7.1 Production of toxin

The containers of the medium are inoculated aseptically with a glass syringe or Pasteur pipettes using one seed culture tube for the inoculation of a single container. The containers are then incubated at $35^{\circ}C \pm 1^{\circ}C$. Such cultivation cannot be considered to be performed initially under anaerobic circumstances, but the highly reducing properties of the medium are sufficient to promote good growth and toxin production. The toxin is harvested after five to seven days' incubation; the average yield of toxin varies between 60 and 80 Lf/ml.

T7.2 Harvesting of toxin

At the end of the incubation period, 10 ml samples are withdrawn from each container and each sample is centrifuged at 3000 rpm for 10 minutes. The supernatant in each sample is checked for toxin (Lf) content. Smears are prepared from the deposit, Gram stained and examined for purity. The contents of the pure containers are filtered through a thick pad of absorbent cotton-wool and pooled together in a stainless-steel pressure tank.

T7.3 Separation of toxin from culture

Separation of the toxin may be carried out before or after detoxification. The yield of toxoid from toxin is similar by either method, but when detoxification takes place in the presence of the organisms more formalin (0.6% v/v) is required. The reason for toxoiding the whole culture is a safety measure to avoid handling large quantities of potent toxin and viable organisms which is dangerous even though the staff may be specially trained in such procedures.

When the toxin is separated from the culture before toxoiding a Seitz filter containing the required number of clarifying and sterilizing pads is sterilized by autoclaving at 126°C for 45 minutes. The filter press must not be fully tightened before sterilization and all valves kept open to allow the free passage of steam during sterilization. The harvest is filtered through the sterile filter under positive pressure not exceeding 10 pounds per square inch (69 kPa). The filtrate is collected in sterile glass or stainless-steel containers of an appropriate capacity.

T7.4 Production of bulk toxoid

Detoxification: When the toxin has been separated from the culture, detoxification is effected by the addition of formalin and incubation of the product at 37°C for some weeks.

The formalin to be used for toxoiding should be as pure as can be obtained, preferably Analar grade, or that conforming to the requirements of the International Pharmacopoeia or other national pharmacopoeias. It should be checked for its formaldehyde content before use (see Appendix T.18).

The procedure adopted is to measure the volume of the toxin by a suitable method and to add sufficient formalin (40% formaldehyde solution) to give a final concentration of about 0.5% v/v. The pH is adjusted to 7.6 with sterile sodium carbonate or bicarbonate solution and the containers incubated at 37°C. The pH is checked after four and seven days, and adjusted again if necessary to 7.6. Incubation is continued for four weeks.

At the end of the incubation period a sample should be taken and the bulk toxoid stored in the cold until the specific toxicity test has been completed (see Appendix T.14). Control tests should be carried out on samples as given under T7.5.

If the sample shows residual specific toxicity, add to the lot an additional quantity of 0.1% formalin and incubate for a further period of two weeks at 37°C, at which time a retest for specific toxicity should be carried out. When toxoiding is complete a sample is taken and the control tests as in T7.5(ii) are applied.

T7.5 "In process" tests or production control tests

When the whole culture is toxoided before purification a sample of the toxin is taken before detoxification for the determination of Lf/ml, but in this case none of the other tests included are T7.5(i) are applicable. After detoxification the tests listed in T7.5(ii) are applicable.

A sterility test is carried out at this time. Experience in the production of the toxoid may allow assessment of this test at a time earlier than the full 14 days incubation period, making it possible to proceed with production after incubation of the sterility samples for 48 to 72 hours. In any event the sterility test must be allowed to remain for the full 14 days.

Where a number of tests are applied at the same point in the production process it is advisable to carry out the tests in such an order that those making the least demands on resources and giving the quickest results should be performed first. These should be shown to be satisfactory before the more demanding and time-consuming tests are undertaken.

T7.5(i) "In process" tests for toxin

The following "in process" tests shall be carried out on the toxin:

I	Sterility	Appendix T.28
II	Determination of Lf content	Appendix T.6
III	Determination of M.L.D. (minimum lethal dose)	Appendix T.7
IV	Determination of M.T.V. (maximum toxin value)	Appendix T.9
V	Determination of Lf/10 dose	Appendix T.8

T7.5(ii) "In process" tests for toxoid

I	Specific toxicity test	Appendix T.14
II	Sterility	Appendices T.20 and T.28
III	Determination of Lf/ml	Appendix T.6
IV	Total combining power	Appendix T.10

T7.6 Purification of toxoid

Although ultrafiltration of toxoid removes some impurities it is nevertheless advisable to apply a further purification procedure to tetanus toxoids.

The simplest and most commonly used procedure involves salting out of the toxoid with ammonium sulfate and the method described in Appendix T.5 is satisfactory. At low ammonium sulfate concentrations relatively impure toxoid is precipitated compared with that precipitated at higher ammonium sulfate concentrations (van Ramshorst, Antonie van Leeuwenhoek (1957) 23, 97). The immunizing potency of the toxoid precipitated by high ammonium sulfate concentrations appears to be very variable. Between these two extremes, toxoid of high purity (usually 1500-2000 Lf/mgN) is obtained. As the optimum concentration of ammonium sulfate varies from batch to batch it is necessary to determine a precipitation curve for each batch (see Appendix T.5). The curve is S-shaped with the purest toxoid precipitated where the curve has the steepest slope. Usually the first precipitation is carried out at 12-15% of ammonium sulfate (the percentage of ammonium sulfate is expressed as grams of solid ammonium sulfate added to 100 ml of the toxoid), and this precipitate which consists of non-specific impurities is discarded. The ammonium sulfate concentration of the supernatant is then raised to approximately 25% or to the concentration as indicated by the precipitation curve in order to precipitate the purest toxoid. This precipitate is processed further as described in Appendix T.5.

Toxoid recovered by this procedure will contain about 60-70% of the original toxoid content with an average purity varying between 1000 and 1200 Lf/mg protein N. Such a toxoid is considered acceptable.

T8. POOLING, STORAGE AND TESTING OF BULK TOXOID

T8.1 Pooling

The volume of an individual batch of toxin or toxoid is usually about 50-100 litres. Before proceeding to ultrafiltration and/or purification it is convenient to pool several

such batches to make about 500 litres (or more, if the facilities permit). If by ultrafiltration or salting out the volume is reduced approximately 1:50, then the resultant concentrated toxoid would be about 10 litres, a convenient quantity to handle for purification by fractional salting out. After purification, the volume of the concentrated and purified bulk toxoid would be still less (6-8 litres), again a convenient volume for the preparation of final vaccine.

T8.2 Storage

As the concentrated and purified toxoids are very stable they can be stored in a refrigerator ($5^{\circ}C \pm 3^{\circ}C$) for some years before further processing but after a long storage period it is advisable to check the Lf content before further processing.

T8.3 Testing of bulk toxoid

Each bulk toxoid after purification shall be subjected to the following tests:

I	Potency test	Appendix T.13
II	Specific toxicity	Appendix T.14
III	Sterility	Appendix T.20
IV	Test for antigenic concentration:	
	(a) Determination of Lf/ml	Appendix T.6
	(b) Determination of total combining power (TCP)	Appendix T.10
V	Purity, Lf/mg protein N	Appendices T.6 and T.15
VI	Thiomersal content	Appendix T.14
VII	Free (residual) formaldehyde content	Appendix T.18
VIII	Test for irreversibility	Appendix T.11

T8.4 Potency test

There are several methods of measuring the potency of tetanus toxoid, but for each the principle is the same. Groups of animals (guinea-pigs or mice) are immunized with graded doses of the toxoid and their responses measured either by determining the antibody titre in the sera or by challenging the animals with a paralytic or lethal dose of toxin.

In order to rule out the variabilities that are inevitably involved in an animal test, similar groups of animals are immunized with a reference preparation that has been calibrated in International Units. By comparison of the responses elicited by the test and reference toxoid the potency of the test preparation may be recorded in International Units.

The suggested method for measuring the potency of tetanus toxoid is described in Appendix T.13.

T9. DILUTING AND FILLING

In some countries, tetanus toxoid is used both as a plain and as an adsorbed toxoid; it is advisable to use the adsorbed toxoid because of its superior immunogenicity.

T9.1 Preparation and testing of fluid toxoid

Fluid tetanus toxoid is prepared by diluting the concentrated bulk which has passed all statutory requirements: specific toxicity, sterility, potency, etc. The final bulk should contain at least 10 Lf/ml (5 Lf per human dose). Where fluid toxoid is used alone the concentration used should be not less than 10 Lf/human dose and in some countries be as high as 20 Lf. Dilution can be made in either normal physiological saline or buffer solutions such as phosphate or borate buffer; in order to maintain the pH of the final product through the storage period buffered saline is to be preferred. Fluid tetanus toxoid in phosphate buffered saline is prepared by the following method.

The Lf content of the bulk concentrated tetanus toxoid is determined and the quantity of the toxoid required for the final bulk is measured out aseptically. If a final bulk of 100 litres is to be prepared the sodium chloride required to make 100 litres of 0.7% sodium chloride (700 g per 100 litres) is weighed out and dissolved in 20 litres of distilled water and 1 litre of one molar phosphate buffer is added. One litre of 1% thiomersal is also added, the volume is made up to about 70 litres with distilled water and the solution is sterilized by filtration (e.g. through a Seitz filter or 0.22 micron membrane). At this stage the required volume of toxoid is added aseptically and the volume made up to 100 litres with sterile distilled water. The pH should be between 6.5 and 6.8 and is adjusted with sterile phosphate buffer solution if necessary. The final bulk is then thoroughly mixed.

T9.2 Adjuvants

Adjuvants such as aluminium phosphate or aluminium hydroxide are used in the preparation of adsorbed toxoids. Adjuvants may be prepared locally or obtained from a commercial source. The latter is especially advisable for aluminium hydroxide gel which is difficult to prepare, and preparations such as "Alhydrogel" are commercially available. Two methods for the preparation of aluminium phosphate are given in Appendix T.20. Calcium phosphate is also used as an adsorbant.

The concentration of aluminium shall not exceed 1.25 mg per single human dose.

T9.3 Preparation and testing of adsorbed toxoid

In the preparation of the final bulk the toxoids are added to the aluminium phosphate, the pH of the mixture is adjusted to 6.0. The mixture is held at this pH for 48 hours, then the pH is readjusted to 6.6 to 7.0. Adjuvant is added to the pertussis component and the pH adjusted to 6.6 to 7.0. The requisite quantities of adsorbed toxoids and pertussis are then pooled, mixed and the pH checked (see Appendix T.23). The pH may be directly adjusted to 6.8 when aluminium hydroxide is used. (The other two components, viz. diphtheria toxoid and pertussis vaccine are omitted when tetanus toxoid (adsorbed) alone is to be prepared.)

The vaccine is merthiolated 1:10 000, taking into account the quantity of merthiolate which entered through the various components.

Where only low grade (type II or III) glass is available it is advisable to add a buffer to the final bulk to prevent the pH rising about 7.0 during the storage period of the vaccine in the final containers.

There are a number of formulations of vaccines. The commonest in use are as follows:

1. Diphtheria toxoid - at least 25 Lf per dose for adsorbed or combined vaccine. It is advisable not to use plain (unadsorbed) diphtheria toxoid alone.

2. Tetanus toxoid (plain toxoid) should contain at least 10 Lf per single human dose; in combination with pertussis the combined vaccine should contain at least 5 Lf of tetanus toxoid per single human dose.

3. Pertussis must contain not more than 20 Opacity Units (O.U.) with a potency of not less than 4 I.U. per single human dose.

The majority of control tests are carried out at this stage when all the ingredients of the vaccine, toxoids (diphtheria and tetanus), pertussis suspension, adjuvant (where applicable) and diluent, have been blended. These tests are:

I	Potency test	Appendix T.13
II	Innocuity test	Appendix T.21
III	Specific toxicity test	Appendix T.14
IV	Sterility test	Appendix T.20

V	Lf content (for fluid vaccine)	Appendix T.6
VI	Thiomersal content	Appendix T.16
VII	pH	Appendix T.17
VIII	Aluminium content	Appendix T.19
IX	Free (residual) formaldehyde	Appendix T.18
X	Test for adsorption	Appendix T.6

T9.4 Filling into final containers

The requirements for filling the final containers shall be as described in the General Requirements for Manufacturing Establishments and Control Laboratories (WHO Technical Report Series (1966) 323) (see Appendix T.29). The volume present in a single bulk container at any time shall determine the batch size. However, the size of the filling lot will be governed by the capacity of the filling equipment to be used and the procedure adopted in filling. If the parent final bulk is distributed in containers of smaller size, each such container shall form a filling lot and be individually labelled as such. If, however, the filling is carried out directly from the final bulk container, the number of final containers filled in one session without a stoppage or break shall be deemed as a final lot or filling lot for all the tests on final containers.

Completely automatic equipment for filling, stoppering and sealing multidose containers is available from reputable manufacturers such as those mentioned in Appendix T.24, item 9. Where such facilities are not available semi-automatic filling equipment comprising automatic filling followed by manual stoppering and automatic sealing may also be obtained.

For single dose containers ampoules of appropriate capacity should be used.

The filling of biologicals must be carried out under strict aseptic conditions as the product once contaminated can neither be recovered nor terminally resterilized. It is, therefore, preferable that filling should be carried out by a completely automated process with least interference by the filling staff. Such completely automatic filling, stoppering and sealing equipment for multidose containers is commercially available (see Appendix T.24). Similarly, automatic ampoule filling and sealing equipment is also available (see Appendix T.24) for filling single dose ampoules. Semi-automatic machines for filling vials followed by manual stoppering and capping or manual stoppering and mechanical capping can also be used. It is preferable, however, to use completely automatic equipment so as to maintain aseptic conditions throughout the operation. Such machines necessarily require the use of vials properly standardized with respect to body diameter, height and dimensions of the mouth so as to be compatible with standard rubber closures. Similar precautions are also required for the dimensions of ampoules to be used in automatic filling.

The quality of glass containers influences the keeping quality of the final product. It is necessary, therefore, to use neutral borosilicate U.S.P. type 1 containers in the interest of quality and safety of the product though U.S.P. type 2 glass may be permissible in some countries.

The quality of rubber closures is also important. Rubber is known to absorb phenols and mercurial preservatives and good quality or coated rubber is needed to prevent this. Sources of rubber should be checked, therefore, for compatibility with the vaccine and its preservative. Butyl rubber stoppers treated with such preservatives as thiomersal or phenol prior to use in the final product are found to absorb a minimum quantity of preservative during storage of the vaccine. The use of butyl or other rubber stoppers which have been shown to be satisfactory is recommended.

The advantage of fully automatic filling and capping equipment is not that it saves labour but that it reduces contamination and thereby prevents unnecessary discarding of valuable vaccine. Stress should be placed on the need for regular maintenance of such equipment.

Prior to filling the vials and stoppers must be washed and sterilized. Many types of equipment are available for this purpose, and a list of some manufacturers is given in Appendix T.24, item 10.

The filling must be done in specially designed rooms or done using a large vertical laminar-flow system which gives good ("Class 100") conditions for sterile work. The names of some manufacturers of such equipment are given in Appendix T.24, item 11. Such laminar-flow systems should be tested from time to time to ensure that they are delivering sterile air. This may be done by exposing nutrient media in Petri-dishes and thereafter incubating them to test for sterility. Alternatively, the contents of the Petri-dishes may be transferred to more easily transportable containers for further incubation.

It is important to check the filling process regularly by filling at least 500 ampoules or vials with a suitable nutrient medium, preferably at the end of the working day. The filled ampoules should be incubated and if more than 1% of them are shown to be contaminated, the filling area, equipment and operating procedure must be investigated and improved.

T9.5 Inspection

Every single or multidose container should be inspected visually under proper illumination to detect the presence of foreign particles. A simple arrangement for this purpose is a fluorescent tube fitted at the top of a black and white background screen and illuminating the background. The fluorescent tube is covered with a shade in order to protect the eyes of the inspection staff from any direct glare. Vials are examined against both white and black background, and vials or ampoules showing foreign particles and defects in closures should be rejected.

T10. TESTS ON FINAL PRODUCTS

T10.1 Tests

The final product after filling shall be subjected to the following tests:

I	Potency test (if not done on final bulk)	Appendix T.13
II	Innocuity test	Appendix T.21
III	Identity test	Appendix T.12
IV	Sterility test	Appendix T.20
V	Thiomersal content	Appendix T.16
VI	pH	Appendix T.17
VII	Aluminium content (where applicable)	Appendix T.19

T10.2 Reporting of data

For each lot of final vaccine a written protocol should be assembled containing all the essential information on the production and testing of the vaccine. It should contain the data of all tests that have been performed and be signed legibly by a responsible member of staff. Apart from these final protocols careful records shall be kept in the laboratory indicating all steps of processing, testing, filling and distribution. All records shall be retained throughout the storage period of the vaccine. The record shall be available for inspection at all times by the national control authorities.

T11. LABELLING AND PACKAGING

T11.1 Labelling

The labelling of tetanus toxoid on the container and package shall comply with the Requirements of Section 8 or Part A of the Requirements for Diphtheria and Tetanus Toxoid (see Appendix T.27).

T11.2 Packaging

The size and type of container and package shall be that most convenient for the conditions of use. It is common to have 10-dose vials, but under some circumstances in which a large number of children may be assembled at a clinic, 25- or 50-dose vials may be more appropriate especially if a jet-injection gun is used. Experiences with jet-injection guns, however, in which adsorbed vaccines are used have given variable results.

Careful consideration must be given to the period over which the contents of a vial may be used. If a physician is giving vaccine to individual children in his surgery and entering the vial on a number of separate occasions, the bacteriostatic agent present in the vaccine must be shown to be capable of suppressing the growth of any organisms introduced into the vaccine during the removal of a single dose of vaccine. It is recommended that a vial once opened should be used on the same day and not be reused on subsequent days. If storage and reuse are absolutely unavoidable, the vial must be kept in a refrigerator at $5^{o} \pm 3^{o}$C and never for more than one working week.

T12. RELEASE

The mechanism for the release of vaccines should be determined by the national control authority. Where there is no national control authority permission to manufacture vaccines and the mechanism for release should be specified by the national health authority.

It is important that only those vaccines that fulfil Part A of the WHO Requirements for Tetanus Toxoid are released for use.

Where the producer is solely responsible for quality control and there is no national control authority, then it is essential that the chief of the quality control laboratory of the producer reports to the director of the institute independently of those involved in the production of the vaccine. The decision concerning the release of a batch of vaccines must be made by the chief of the control laboratory.

T13. RETAINED SAMPLES

At the time of release of a lot of vaccine the manufacturer must set aside and refrigerate enough samples to serve two essential purposes:

(i) to enable an investigation to be carried out in the event of a complaint from a user concerning the particular lot. It is advisable, therefore, to set aside sufficient samples to allow for a full test of the final vaccine to be repeated;

(ii) to ensure that the product has been stored correctly and that the potency has been maintained throughout the storage period.

The storage of retained samples occupies refrigeration space that may be limited, but only if it is known that the complete lot has been distributed, used or destroyed at the end of the expiry period should such samples be discarded. It will be useful from time to time to keep some samples beyond the expiry date, and to check on the keeping properties, particularly for potency.

T14. SURVEILLANCE OF VACCINES

The surveillance of the use of vaccines is the responsibility of the National Government Health Authority. Such surveillance falls into two categories:

T14.1 Safety

Although every attempt is made in the laboratory to ensure that vaccines are safe by subjecting all lots to tests for freedom from abnormal toxicity, a constant watch should nevertheless be kept for adverse reactions in children.

There are contraindications to the use of all vaccines, and an investigation of all adverse reactions should be made particularly if they appear to be lot-related.

T14.2 Efficacy

Different populations differ in their antibody responses to vaccines, and it is essential to ensure that the community is giving adequate antibody responses to all components of the vaccines used. Many factors such as nutritional status, intercurrent infections and the age of the children have an effect upon the responses. A paired blood sample before and after vaccination should be taken from a number of the

vaccinees and titrated to measure the antibody responses in order to have some idea of the effectiveness of the vaccination campaign. Furthermore, it is advisable to obtain blood samples from the same children two to five years later to determine how much antibody (and thereby protection) has been maintained.

T15. WHO REQUIREMENTS

Throughout the manual reference has been made to the requirements specified in the Requirements for Diphtheria Toxoid, Pertussis vaccine, Tetanus Toxoid and Combined Vaccines (1978). The part of these Requirements which refers to Tetanus Toxoid is reproduced in Appendix T.27 of this manual.

T16. SUMMARY PROTOCOLS

A summary protocol for recording the results of tests on a particular final lot of toxoid is included in the manual (see Appendix T.25). The tests specified follow closely those included in the WHO Requirements and the protocol is intended for the reporting of data to the national control authority or to the authorities in other countries to whom the vaccine may be exported. The tests specified are in such detail that inspection of the protocols should give an indication of whether the lot meets the WHO Requirements in every particular.

LIST OF APPENDICES

APPENDIX T.1

MEDIA

The most commonly used medium for the preparation of tetanus toxoid is that developed by Mueller & Miller. The chief constituents of this medium are an enzymic digest of casein and beef heart infusion as the sources of nitrogen and carbon together with glucose, salts and other accessory growth factors such as vitamins, amino-acids and uracil.

The yield of toxin in this medium depends mainly upon the sources of nitrogen. N.Z. Case manufactured by Sheffield Farms was found to be suitable in the absence of beef heart infusion and is being used by many laboratories, but a batch to batch variation occurs. Therefore, only a pre-tested batch found to yield 50 Lf per ml of medium or more should be used. In addition to N.Z. Case, other sources of enzymic digests of casein developed specially for toxin production are commercially available. Tryptone T of Oxoid Laboratories and Tryptone T.S. of Bengal Immunity & Co., Calcutta, India, have also been found to give satisfactory results.

The original medium of Mueller & Miller contained beef heart infusion which is also a source of bovine protein. It is generally desirable to reduce the quantity of such heterologous proteins in order to minimize its likelihood of hypersensitivity to the final product but it has been found that the variability of tryptic digests of casein may necessitate the inclusion of beef heart infusions.

A protein-free medium was developed by Stone[1] and was improved by Latham[2]. The modification by Latham has also been adopted by many laboratories. This medium also

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[1] Stone, J. L. (1953) Appl. Microbiol., I, 166

[2] Latham, W. V. et al. (1962) Appl. Microbiol., 10, 146.

contains essentially the same constituents excepting beef infusion. For good yields of toxin, however, a strain of C.tetani adapted to this medium is required.

The improved medium of Mueller & Miller (Mueller, J. H. & Miller, F. A., J. Bact., 1954, 67, 271) and Latham's modification are given below. The method for the preparation of Mueller & Miller's medium is given in detail. A similar procedure is also followed in the preparation of Latham's medium.

Preparation of Mueller & Miller's medium

The improved medium as described by Mueller & Miller (J. Bact., 1954, 67, 271-277) with some modifications may be used.

The composition of the medium is as follows:

1.	N.Z. Case[1] 10% solution (pancreatic digest of casein or Tryptone T or TS)		250 ml
2.	Meat infusion		50 ml
3.	Glucose		11.0 g
4.	Sodium chloride		2.5 g
5.	Disodium hydrogen phosphate (Na_2HPO_4)		2.0 g
6.	Potassium dihydrogen phosphate (KH_2PO_4)		0.15 g
7.	Magnesium sulfate ($MgSO_4 7H_2O$)		0.15 g
8.	Uracil (25 mg%)	(250 mg/l)	10 ml
9.	Tyrosine (10%)	(100 g/l)	5 ml
10.	Cystine (10%)	(100 g/l)	2.5 ml
11.	Riboflavin (25 mg%)	(250 mg/l)	1.0 ml
12.	Calcium pantothenate (100 mg%)	(1 g/l)	1.0 ml

[1] N.Z. Case is a proprietary name of Sheffield Farms.

Appendix T.1

13.	Thiamine (25 mg%)	(250 mg/1)	1.0 ml
14.	Pyridoxine (25 mg%)	(250 mg/1)	1.0 ml
15.	Biotin (1.25 mg%)	(12,5 mg/1)	0.2 ml
16.	$FeSO_4$ $7H_2O$		0.40 mg
17.	Distilled water to make up the volume to 1 litre		

To 500 ml of distilled water att 11.0 g of glucose, 2.5 g of NaCl, 2.0 g of Na_2HPO_4, 0.15 g of KH_2PO_4 and dissolve by stirring.

0.15 g of $MgSO_4$ $6H_2O$ is dissolved in 50 ml of water and added to the glucose salt solution. The vitamins and amino-acid solutions are added in the following order:

Uracil 10 ml;

Tyrosine 5 ml;

Cystine 2.5 ml;

Riboflavin 1 ml;

Ca. pantothenate 1 ml;

Thiamine 1 ml;

Pyridoxine 1 ml;

Biotin 0.2 ml;

$FeSO_4 7H_2O$ dissolved in 50 ml of distilled water.

Then 50 ml of meat infusion and 250 ml of N.Z. Case solution are added. The solution is thoroughly stirred for proper mixing. The volume is then made up to 1 litre with distilled water. The mixture is again stirred. pH is then adjusted to 7.2 to 7.3 with 10 N sodium hydroxide.

The stock solutions required for the above are made as follows:

N.Z. Case. 100 g of N.Z. Case are dissolved in hot distilled water and made up to 1000 ml and cooled. 1.5 g of activated animal charcoal is added to this, shaken well to mix and filtered after 20 minutes.

Meat infusion. 2000 ml of distilled water are added to 1 kg of meat; kept in cold overnight. Next day it is boiled for 30 minutes and filtered. It is autoclaved for 20 minutes at 121°C.

Uracil. To 200 mg of Uracil 22.4 ml of concentrated hydrochloric acid are added in a glass mortar and triturated with the pestle to dissolve. The volume is then made up to 800 ml with distilled water.

Tyrosine. 50 g of Tyrosine are dissolved in 25% HCL and the volume is made up to 500 ml with distilled water.

Cystine. 50 g of cystine are dissolved in 25% HCL and the volume is made up to 500 ml with distilled water.

Riboflavin. 10 mg of riboflavin are added to 40 ml of 25% alcohol and dissolved by heating in a water bath at 56° for 30 minutes. Fresh solution is always used.

Calcium pantothenate. 100 mg of calcium pantothenate are dissolved in 100 ml of 25% alcohol.

Thiamine. 25 mg of thiamine are dissolved in 100 ml of 25% alcohol.

Pyridoxine. 25 mg of pyridoxine are dissolved in 100 ml of 25% alcohol.

Biotin. 10 mg of biotin are dissolved in 800 ml of 25% alcohol.

Appendix T.1

After all the ingredients are mixed the medium is distributed into the containers. They are covered with a cotton-wool pad in between two layers of hydrophil gauze and autoclaved at 121°C for 20 minutes. The containers are taken out of the sterilizer and quickly cooled in running tapwater or cold water and taken immediately for inoculation.

An alternative medium with almost the same constituents of the medium but without meat infusion is used also in some of the laboratories. The composition of the medium is given below:

1.	Tryptone T or N.Z. Case, or Tryptone TS	30.0 g
2.	Glucose	8.0 g
3.	Sodium chloride	2.5 g
4.	Magnesium sulfate ($MgSO_4$ $7H_2O$)	0.1 g
5.	Cystine	0.25 g
6.	Calcium pantothenate	3.0 mg
7.	Potassium chloride (KCl)	0.1 g
8.	Nicotinic acid	0.75 mg
9.	Riboflavin	0.25 mg
10.	Pyridoxine	0.25 mg
11.	Vitamin B12	0.05 µg
12.	Biotin	5.0 µg
13.	Thiamine	0.25 mg
14.	Para amino benzoic acid	1.0 mg
15.	Ferric chloride ($FeCl_3 6H_2O$)	0.032 g
16.	Distilled water to make up to 1 litre	

APPENDIX T.2

PRODUCTION IN FERMENTERS

A typical fermenter is a cylindrical vessel constructed of high-grade stainless-steel, preferably AISI 316. All contact parts including welds must be polished. The dimensions of the body of the fermenter depend on the size of the lot which is to be prepared. A "small fermenter" is used for a lot size of 50-60 litres, and is approximately 40-45 cm in diameter and 60-70 cm in height. In determining the dimensions of the fermenter one must bear in mind the dimensions of the available autoclave, if the fermenter is to be sterilized by autoclaving (heating up time of half an hour followed by one hour at 125-130°C for an empty fermenter).

The fermenter is jacketed, and tap water, the temperature of which is automatically regulated, flows through the jacket thereby controlling the temperature of the culture in the fermenter as required.

Some design features

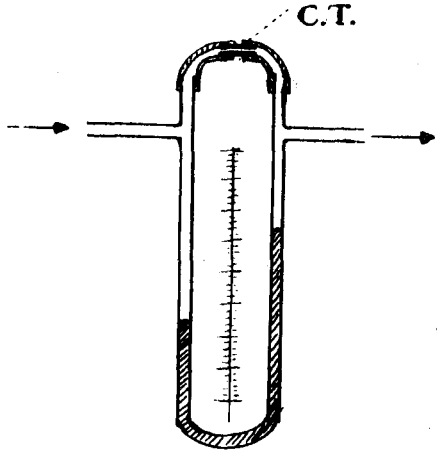
Air inlet and air outlet, media inlet and sampling tube are designed for connection to rubber or, preferably, heavy wall silicon tubing. The air for aeration of the fermenter must be sterile, which is achieved by filtration through suitable filters, e.g. "Line Filters" manufactured by MICROFLOW (see Appendix T.24, item 22). The outlet air should be handled with care because it is contaminated: it should pass through bottles containing disinfectants and/or through terminal filters electrically heated to about 250°C.

The interior of the fermenter may be viewed through a viewing port, a toughened glass disc, 7.5 - 10 cm diameter, fitted in a threaded or bolted holder secured with O-rings; cement must not be used to secure the viewing disc. The interior of the fermenter can be illuminated through a second, similar port. If two ports are included in the design they are usually sited opposite each other on a diameter, either on the lid or shoulder of the fermenter.

Appendix T.2

The flow of air for aeration must be monitored and a simple way of doing this is to insert a differential flow meter in the air line, before the sterile filter. The design of this meter is simple (see Fig 1) and it can be constructed in the laboratory.

FIG.1. DIFFERENTIAL FLOW METER FOR AIR FLOW



The length and bore of the capillary tube (CT) are chosen so that the desired rate of flow can be read esaily on the scale. The instrument must be calibrated in litres/minute by blowing air into an inverted 10-litre bottle filled with water, the neck of which is immersed in water.

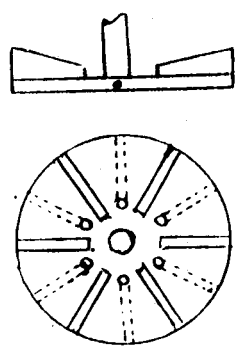
The temperature of the culture in the fermenter is controlled by regulating the temperature of the water flowing through the jacket. The simple way to achieve this is to insert in the water inlet to the jacket an electric immersion heater, which is automatically switched off and on as commanded by a contact thermometer or other sensitive thermostatic device (thermistor probe) inserted into the thermometer well of the fermenter.

Appendix T.2

Mixing and aeration in a fermenter is extremely important in order to obtain good growth of cells. Speed of mixing (rpm), size and type of impeller and rate of aeration (litre/minute) greatly affect growth and must be optimised for each size and design of fermenter.

"Vortex mixing" as described by Chain et al., Bulletin of the World Health Organization (1952), 6, 73 is often used. The impeller as sketched in the accompanying Fig. 2 is located about 15 cm above the bottom of the fermenter, and is driven by direct drive of a (three-phase) electric motor.

FIG. 2. SKETCH OF THE IMPELLER IN A FERMENTER

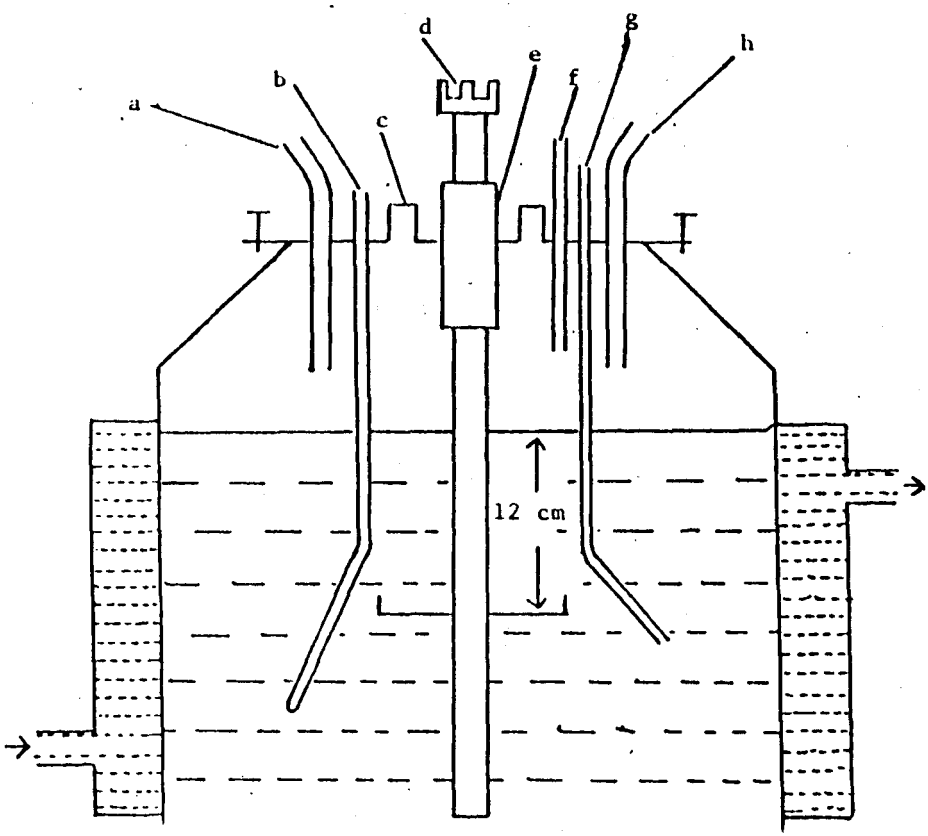


For production of pertussis in fermenters it is possible and convenient to use a magnetic drive for stirring (see for example Cameron & Godfrey, Magnetic Drives, Biotechnical and Bioeng. (1974) Symp. No 4, 821-835).

A diagrammatic view of a typical fermenter, as used at Connaught Laboratiries Ltd., Toronto, Canada, is represented in Fig. 3:

Appendix T.2

FIG. 3



a - Air inlet

b - Thermometer well

c - Viewing port

d - Coupling for drive shaft

e - New Brunswick Co. Shaft-seal assembly

f - Media inlet

g - Sampling tube

h - Air outlet

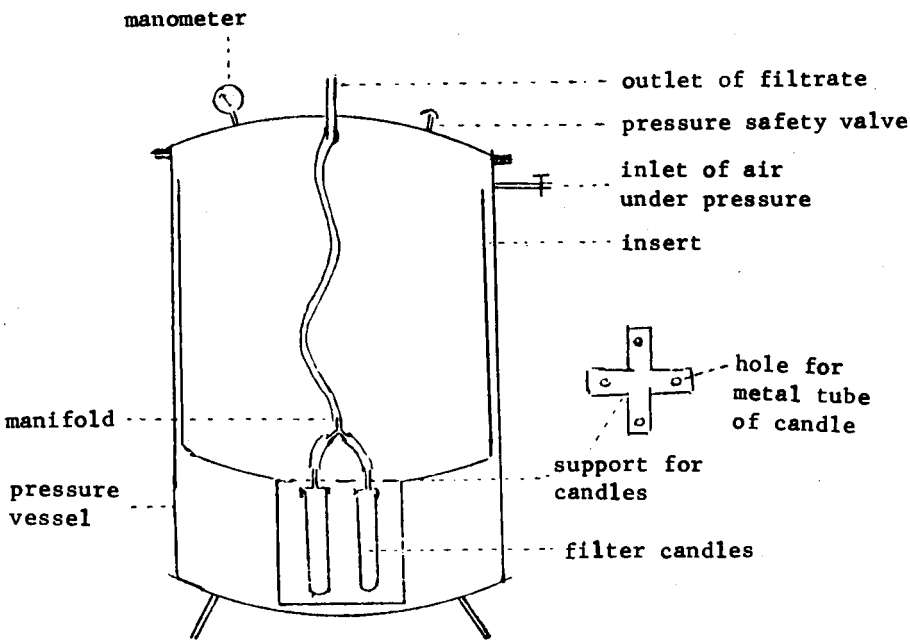
For further details concerning the construction and functioning of this fermenter, see Stainer & Scholte, Biotechn. and Bioeng. Symp. (1973), No. 4, 283-293.

APPENDIX T.3

FILTRATION OF FERMENTER HARVEST

The manual has suggested filtration through multisheet filters or Meta filters but the following method through filter candles under pressure is also effective.

A vertical cylindrical pressure vessel of 150-250 litres capacity is needed and an old type autoclave is quite suitable. An insert made of high quality stainless-steel, polished on the inner surface with all welds also highly polished, is placed inside as shown in the figure. The lower part of the insert is constricted and forms the actual filtration chamber.



Appendix T.3

Four or more filter candles, attached to a cross-like support are placed in the chamber. Suitable candles are Berkefeld filter candles, type N (the source of Berkefeld filter candles is given in Appendix T.24).

A piece of heavy wall rubber tubing is used to connect the candles to a manifold (glass or stainless-steel), which in turn is connected by a single piece of tubing to the outlet. This outlet can also be located on the side wall of the vessel. If it is located on the lid (as in the figure), the piece of tubing connecting it to the outlet should be longer (as represented in the figure), so as to allow for the opening of the lid of the vessel.

Procedure: the bacterial sludge is poured into the inner jacket, some filter aid is added (e.g. 2-3% of Hyflo Super Cell from Johns Manville Co.), the contents mixed by stirring, the vessel covered and the air pressure applied. Initially a low pressure of 0.1-0.2 bar (kp/cm^2) should be used and the pressure is slowly increased to 1.0-1.5 bar (kp/cm^2) or more.

The filtrate can be collected separately, or the outlet for the filtrate on the pressure vessel can be connected directly to a sterile filtration unit, so that clarification and sterilization are performed simultaneously. A flow rate of about 60 litres in one to two hours can be achieved.

The dimensions of the lower part of the insert (filtration chamber), the number of candles and the amount of filter aid should be adjusted so that when the filtration is finished, the chamber is almost filled with bacterial mass and Hyflo.

It is important to ensure that the whole inner vessel with its contents are sterile before being discarded.

APPENDIX T.4

ULTRAFILTRATION OF TOXOIDS

Ultrafiltration is carried out by means of collodion membranes supported on filter candles.

(a) Preparation of Palodion solution

Two thousand four hundred millilitres of glacial acetic acid is poured into a 10-litre bottle which is placed on a rocking apparatus (or rocked manually) preferably in an incubator at 35°C to 38°C to speed up the dissolution of parlodion.

From time to time pieces of parlodion (Mallinckrodt Purified Pyroxylin) are added to the acetic acid and rocking continued until about 210 g has been added. A rubber stopper is tied into the neck of the bottle, and rocking is continued for several days until the parlodion is dissolved to yield a clear viscous solution.

(b) Preparation of candles.

Ceramic filter candles (e.g. Berkefeld filter candles, type N, 5 cm in diameter and 25 cm long, from the firm Berkefeld Filter, 3100 Celle, Federal Republic of Germany) are cleaned with very fine sand paper, after which the ceramic part should not be touched by hand otherwise the membrane will not stick correctly to the greasy surface produced by contact with hands. The parlodion solution is poured into glass cylinders, and after waiting for at least 30 minutes until the smallest air bubbles have disappeared the candle is dipped into the parlodion solution for one to two minutes; a small vacuum of 25-30 Hg is applied to the candle. The candle is then taken out of the cylinder, turned upside down and by gently rocking while still under vacuum, the parlodion solution is distributed evenly on the surface of the candle. The candle is then immersed in cold tapwater and left until the whole batch of candles, up to 30 or more, has been prepared. The immersion in water results in the formation of a collodion membrane with ultrafiltration properties.

Appendix T.4

The candles are now washed free from acetic acid. This is done in the same apparatus as that used for the ultrafiltration except that in the washing cycle pure water is used instead of toxoid (see figure). The washing is continued for a few days until the ultrafiltrate does not show any acidity (as measured by indicator paper or pH meter).

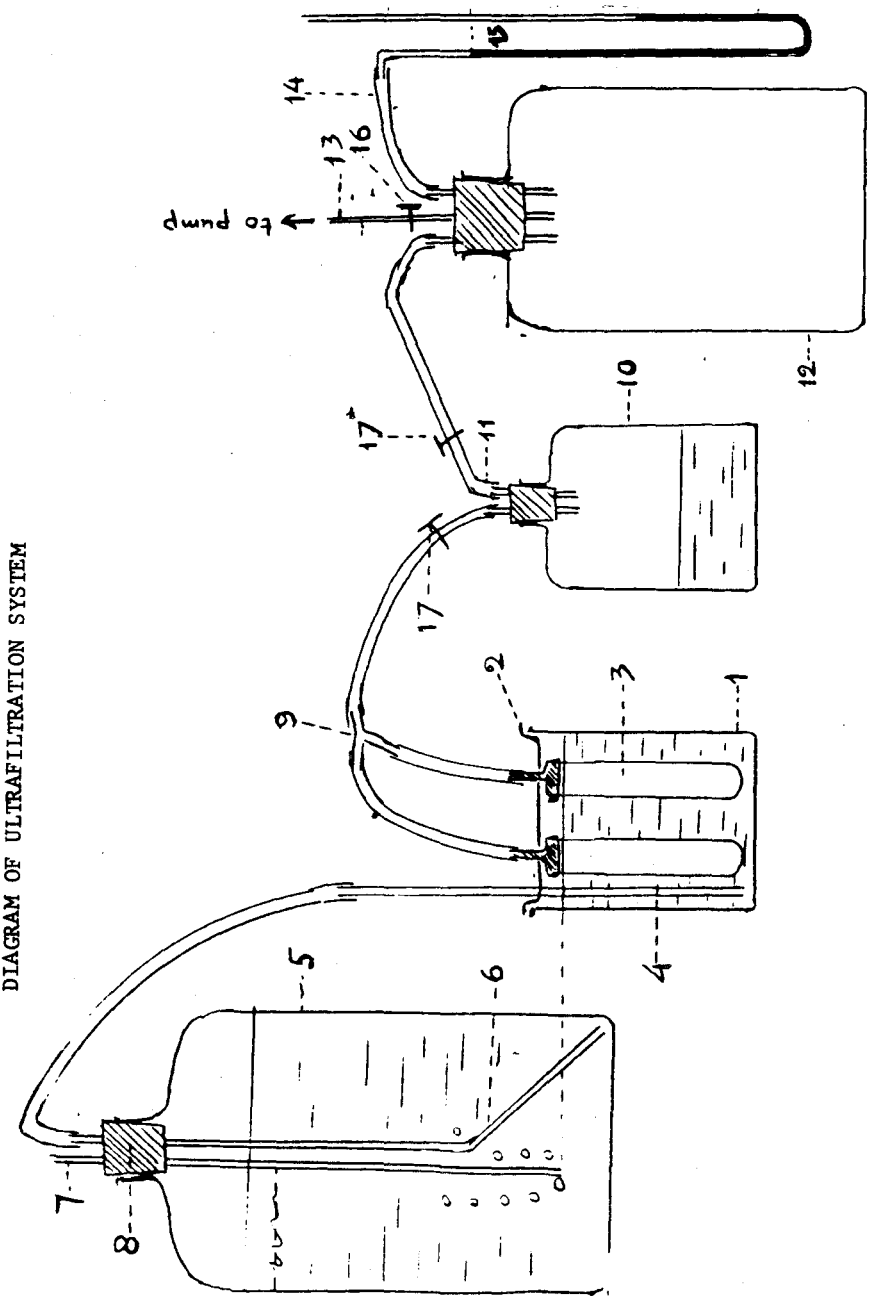
(c) Ultrafiltration procedure

(In the description the numbers refer to the numbers shown in the figure.)

The ultrafiltration cell (1) is a container of any shape (a square cross section of 20 x 20 cm, height 30-35 cm is suitable), made of glass which is preferred to stainless-steel for observing the concentrated toxoid. The cell is covered with a stainless-steel support plate (2), carrying the candles (3). One candle is fixed into each hole in the support plate. For the cell of the above size, the plate has nine holes so that the cell consists of a "battery" of nine candles. In the cell there is also a glass or stainless-steel tube through which the toxoid flows into the cell from a large "Mariotte flask" (5) through the tube (6) which reaches to the bottom of the flask. This flask is also fitted wi with a tube (7) open to the atmosphere through which the air bubbles into the flask as the toxoid flows out. When stoppered tightly (8) the lower end of this tube (7) determines the level of the toxoid in the cell. From the cell (1) the ultrafiltrate passes into the candles (3) and is then collected in the collecting flask (10) (usually a 10-litre bottle) via the manifold (9) and the collecting tube. Another tube (11) leads from this bottle into a "vacuum container" (12) which may be any kind of container large and strong enough (100-200 litres) to hold a partial vacuum of 25-30 cm Hg for some time, e.g. overnight. From this container (12) a second tube (13) leads to a pump (an ordinary water-jet pump is suitable) whilst a third tube (14) is connected to a mercury manometer (15) or similar device to measure the partial vacuum in the system.

Appendix T.4

DIAGRAM OF ULTRAFILTRATION SYSTEM



Appendix T.4

The capacity for ultrafiltration is dependent on the number of candles and the rate of filtration which is approximately 1 litre per candle per 24 hours. It is usual to have several batteries with a common Mariotte flask (5) and vacuum container (12) but with separate collecting flasks (10). If all flasks and containers are adequately stoppered it is sufficient to evacuate the system once in 24 hours, and then to close the valve (16). In this way ultrafiltration will proceed automatically day and night. Once or twice in each 24 hours the collecting flask (10) is opened without breaking the vacuum in the remainder of the system by closing the valves or clamps (17). The ultrafiltrate is tested for traces of protein with trichloroacetic or sulfo-salicylic acid and if no protein is present, the ultrafiltrate is discarded. If protein is found, any defective candles must be located, replaced and the ultrafiltrate recycled. A faulty candle is detected by isolating each candle, taking a sample of the liquid from inside and testing for protein. The concentrated toxoid is recovered from the cell (1) in which there is a 50-fold or greater concentration.

The whole procedure is difficult to perform aseptically. To reduce contamination thiomersal is added (1:10 000) and the toxoid is mixed and covered with a layer of toluene. The whole apparatus is placed in a cold room at $+5^{\circ}C \pm 3^{\circ}C$. Usually 500 litres of filtrate can be reduced to a volume of 10 litres through a battery of 27 candles in 12 to 14 days.

APPENDIX T.5

PURIFICATION OF TETANUS TOXOID

The purification of tetanus toxoid is carried out in two stages. The crude toxoid is first passed through an ultrafilter and the concentrated toxoid is then subjected to fractional precipitation with ammonium sulfate.

Ultrafiltration (see Appendix T.4)

Different types of ultrafilters are available commercially but they are expensive especially for a small-scale production unit. For such a production unit, however, an ultrafiltration assembly can be made in the laboratory.

Fractional precipitation with ammonium sulfate

Determining the curve of precipitation

The optimum concentrations of ammonium sulfate for precipitating impurities and specific toxoid should be determined by a pilot experiment. For this purpose the pH of the concentrated toxoid is adjusted with ammonium hydroxide solution to 7.0 if it is acidic, or with dilute hydrochloric acid if it is alkaline and 10 ml of this toxoid is pipetted into each of 13 tubes. To these tubes increasing quantities of recrystallized solid ammonium sulfate are added to give the following concentrations: 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30%. The tubes are shaken to dissolve the ammonium sulfate and left at room temperature overnight. The next day the tubes are centrifuged, the supernatant is decanted into separate tubes and the precipitate is dissolved in distilled water and the volume is made up to 10 ml. The Lf value of each precipitate is determined. A graph is plotted with concentrations of ammonium sulfate on the x axis and Lf values of the corresponding precipitate on the y axis. The

Appendix T.5

curve is "S" shaped and shows the concentration at which no appreciable toxoid is precipitated as well as the optimum concentration at which maximum amount of toxoid is precipitated. The lower asymptote of the curve indicates the best concentration for the precipitation of impurities with minimal loss of toxoid, whilst the upper asymptote indicates the lowest concentration of ammonium sulfate at which the toxoid can be precipitated in maximal yield.

These concentrations found in the pilot test are applied to the bulk toxoid which is then purified. The volume of toxoid is measured and solid ammonium sulfate added to give the concentration required for the beginning of the straight line. The mixture is left overnight and centrifuged the next day. The supernatant is collected and the precipitate is discarded.

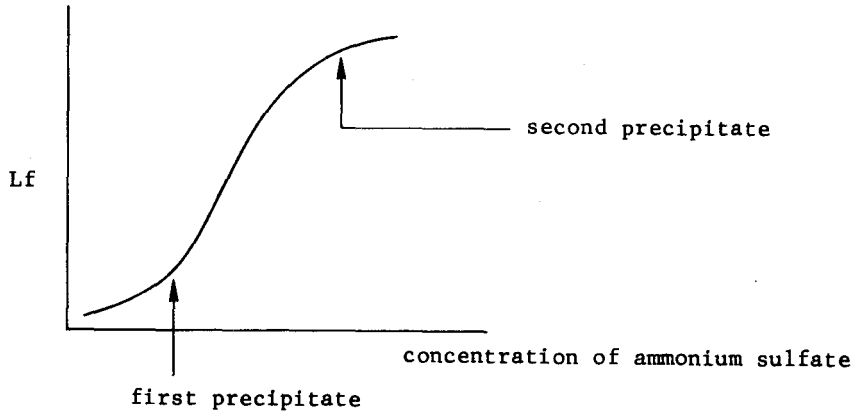
Second precipitation

The ammonium sulfate concentration of the supernatant is raised to the value required for the end of the straight line of the precipitation curve by adding solid ammonium sulfate. The mixture is well shaken and left at room temperature overnight and centrifuged the next day discarding the supernate. The precipitate is dissolved in a minimal volume of distilled water and dialysed in cellophane tubes against distilled water in the cold until all the ammonium sulfate has been removed. Distilled water is changed every day for the first two days and, thereafter every two days for eight days in order to complete the dialysis of ammonium sulfate.

The dialysed toxoid is then sterilized by Seitz filtration. To the filtrate thiomersal is added to give a final concentration of 0.01% and stored in the cold.

Appendix T.5

CURVE OF PRECIPITATION OF TOXOID BY AMMONIUM SULFATE



APPENDIX T.6

DETERMINATION OF Lf/ml

The flocculation test for tetanus toxin is not as simple as that for diphtheria toxin because multiple zones of flocculation occur with tetanus antitoxin. Therefore, a reference antitoxin giving a specific zone of neutralization of the toxin has to be prepared and standardized against a known standard antitoxin obtained from a reputable source.[1] The reference antitoxin may be adjusted to contain 100 Lf/ml.

[1] A source of tetanus antitoxin can be suggested by the WHO Secretariat (Chief, Biologicals).

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For the determination of the Lf value of an unknown sample of tetanus toxin or toxoid, increasing volumes of the reference antitoxin are pipetted into a series of flocculation tubes and the volume is made up to 1 ml with normal saline. To each tube is then added 1 ml of the toxin or toxoid under test and properly mixed by shaking. The tubes are then put in a water bath held at 50° and placed in such a way that only one-third of the tube dips below the water level. The tubes are observed continuously and the tube which shows flocculation is noted. The Lf value of the toxin or toxoid is calculated from the concentration of toxin or antitoxin contained in this tube.

Example. Reference antitoxin contains 100 Lf/ml

Tube No.	1	2	3	4	5	6	7	8
Reference antitoxin ml	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
Normal saline	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0
Toxin lot x	1	1	1	1	1	1	1	1
				F_3	F_1	F_2		

F_1, F_2 and F_3 show the order of flocculation, F_1 being the first to flocculate and F_3 the last. The tube which flocculates first contains the optimum amount of tetanus antitoxin neutralizing the toxin. In the example above tube No. 5 containing 0.7 ml of reference antitoxin equivalent to 70 Lf has flocculated first with 1 ml of toxin. Thus 1 ml of toxin contains 70 Lf. The time required for flocculation (Kf) should be noted.

Appendix T.6

If a more precise result is desired, one can repeat the flocculation test using smaller range (e.g. 30-50 Lf) with increases of 2 Lf from tube to tube.

Blend flocculation

The measurement of very low concentrations of toxoid is best carried out by the method of blend flocculation. This can be performed in many ways but always involves comparison of the Lf value of a known toxoid and that of a mixture of the sample with that toxoid.

For example:

The Lf content of known toxoid (50 Lf/ml) is checked with an additional 1 ml of saline added to each tube.

Tube	A	B	C	D	E	F
Antitoxin added (Lf units)	45	50	55	60	65	70
Antitoxin added (ml)	0.45	0.5	0.55	0.6	0.65	0.7
Saline added (ml)	1.55	1.5	1.45	1.4	1.35	1.3
Known toxoid added (ml)	1.0	1.0	1.0	1.0	1.0	1.0
Flocculation sequence	2	1	3			

Estimated value of known toxin 49 Lf units/ml. At the same time flocculate known toxoid 50 u/ml with 1 ml of unknown toxoid added to each tube.

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Tube	A	B	C	D	E	F
Antitoxin added (Lf units)	45	50	55	60	65	70
Antitoxin added (ml)	0.45	0.5	0.55	0.6	0.65	0.7
Saline added (ml)	0.55	0.5	0.45	0.4	0.35	0.3
Known toxoid (ml)	1.0	1.0	1.0	1.0	1.0	1.0
Unknown toxoid (ml)	1.0	1.0	1.0	1.0	1.0	1.0
Flocculation sequence		3	1	2		

Estimated value for known toxoid + unknown toxoid 56 Lf units.

Contribution of unknown toxoid 56 - 49 = 7 Lf units.

This method is also used when, in the case of tetanus toxoid there is doubt whether the flocculation seen represents the true value of the material (tetanus may show several flocculation zones only one of which is a guide to potency).

When a known toxoid and an unknown toxoid are flocculated together the mixture will flocculate as the sum of their values if they are homogeneous. If different zones are involved they will produce a confused pattern with two flocculation maxima.

Test for non-adsorbed toxoid

The detection of non-adsorbed toxoid is done by centrifugation of a sample of vaccine and determination of the quantity of toxoid in the supernatant by blend flocculation as described above. The quantity of non-adsorbed toxoid should be less than 20% of the total toxoid added to the adjuvant.

APPENDIX T.7

DETERMINATION OF THE MINIMUM LETHAL DOSE OR M.L.D.

The M.L.D. of tetanus toxin is that amount of toxin which when injected into mice of about 20 g in weight kills the majority in four days. For determining the M.L.D. the following method is recommended.

One ml of toxin is added to the tube No.1 and well mixed. One ml from tube No. 1 is transferred to tube No. 2 and well mixed. One ml from the previous tube is transferred to the next tube until tube No. 6 is reached. From tube No. 6, 5 ml of mixture is transferred to tube No. 7 giving a dilution of 1/2 000 000. One ml of dilutions from tube Nos. 4 to 7 are inoculated into mice. If all the mice or at least two-thirds of the mice die on or about the fourth day when given the toxin from tube No. 6 the M.L.D. of the toxin is taken as 10^{-6} or 1/1 000 000.

Example

Tube No.	1	2	3	4	5
Volume of peptone water	9	9	9	9	9
	1 ml	1 ml	1 ml	1 ml	1 ml

6	7
9	5
5 ml	

Appendix T.7

Tube No.	1	2	3	4	5
Dilution of toxin	1/10	1/100	1/1000	1/10 000	1/100 000

Tube No.	6	7
	1/1 000 000	1/2 000 000

Tube No.	4	5
Mice dying / Mice injected	3/3	3/3

Tube No.	6	7
	2/3 M.L.D.	0/3

APPENDIX T.8

DETERMINATION OF L+/10 DOSE

The L+/10 dose of a toxin is the smallest amount of that toxin which when mixed with one-tenth of an International Unit of antitoxin and injected into mice kills the mice on or about the fourth day after injection. For this purpose the standard antitoxin is diluted to contain 0.5 IU/ml, i.e. if the standard antitoxin contains 10 IU it is diluted 1:20 to give 0.5 IU per ml.

One ml of this diluted antitoxin is put into each of four tubes. Different quantities of toxin under test are added to it and the volume is made up to 2 ml by the addition of the requisite amount of saline and the contents mixed.

Appendix T.8

The mixtures are kept for one hour at room temperature and 0.4 ml of each mixture injected into each of three mice each weighing 20 ± 2 grams. The mice are observed for four days and the mixture killing the mice on or about the fourth day contains one L+/10 dose of toxin. Similarly, L+/20 or L+/40 doses can be determined by injecting 1/20th or 1/40th of a unit of standard antitoxin.

An acceptable toxin contains 500 or more L+/40 doses of toxin. L+/10 or L+/20 or L+/40 doses grade the toxins quantitatively.

APPENDIX T.9

DETERMINATION OF MAXIMAL TOXIN VALUE (M.T.V.)

(van Ramshorst, Antonie van Leeuwenhoek (1954), 20, 17.) This value is obtained by mixing constant amounts of toxin with increasing amounts of antitoxin in the same way as in the flocculation reaction and injecting 0.5 ml of the mixtures subcutaneously into mice. The mixture which just fails to produce symptoms of clinical tetanus in mice contains equivalent amounts of the toxin and antitoxin which have neutralized each other. This method is a check on whether the correct flocculation zone has been used for the determination of Lf.

Appendix T.9

Example

Tube No	1	2	3	4	5	6
Reference antitoxin ml (100 IU/ml)	0.5	0.6	0.7	0.8	0.9	1
Toxin in ml	1	1	1	1	1	1
Normal saline	0.5	0.4	0.3	0.2	0.1	0
Mice dying mice inoculated	3/3	3/3	3/3	0/3	0/3	

The tubes are well mixed and held at room temperature for one hour and 0.5 ml of each mixture is injected subcutaneously into three mice. The mice are observed for four days. If all the mice for tube No. 3 die and for tube No. 4 survive without any symptoms of tetanus, the maximal toxin value is 80 units/ml.

APPENDIX T.10

TOTAL COMBINING POWER TEST (TCP)

A suitable reference toxoid which is known to produce an adequate response in laboratory animals as measured by the potency test when used in vaccine should be selected for use in this test. Each laboratory should select a reference preparation which would be a batch of vaccine that had passed the potency test.

Dilute the test sample with buffered saline to a concentration of 2 Lf/ml. Dispense into a series of six tubes 0.3, 0.36, 0.45, 0.55, 0.65 and 0.8 ml respectively of diluted toxoid. Dispense a similar series of doses of the laboratory

reference toxoid into a second series of tubes. Add 0.5 ml of a solution containing 4 IU of tetanus antitoxin per ml to each tube. Incubate at 20-25°C for 30 minutes. Add 0.5 ml of a solution containing 2 units equivalent of tetanus toxin per millilitre to each tube and incubate at 20-25°C for 60 minutes.

Inject the whole contents of the first tube subcutaneously into a mouse weighing 18-22 g. Inject the contents of the other tubes into other, similar mice. Observe the mice for four days and record the incidence of tetanus paralysis and death.

A mixture which causes the death of a mouse in less than four days contains more than 1 TCP (Binding Unit) of toxoid. A mixture which allows survival of a mouse for more than four days contains less than one binding unit. The end point representing equivalence is death of the mouse at four days exactly.

The test is repeated using volumes of the same initial dilutions selected from the range 0.27, 0.33, 0.4, 0.5, 0.6, 0.7, 0.9 millilitres so that the end point of the first test falls in the middle of the range selected for the second test. If the repeat test conforms with the first test, the combined result may be accepted. If the tests disagree further, repeats must be carried out. The ratio of $\frac{TCP}{Lf}$ for the test material should be not less than 80% of that of the reference toxoid.

The TCP of the toxoid is the reciprocal of the volume of toxoid dilution found to be equivalent to 1 IU.

APPENDIX T.11

TEST FOR IRREVERSIBILITY OF TOXOID

Procedure

A sample of purified toxoid is diluted in saline containing 0.01% thiomersal to give 300 ml of 10 Lf per ml toxoid. Aliquots of 100 ml of the diluted toxoid are held at 4^{o}, 20^{o} and 32^{o} for six weeks. 5 ml of each sample is injected subcutaneously into each of five guinea-pigs weighing between 280 and 300 mg, 2.5 ml on each side of the abdomen. The pigs are weighed on the first, third and seventh day following injection, then weekly for a further three weeks. The guinea-pigs are examined daily for any evidence of tetanus toxin poisoning.

Criteria for acceptance

The animals should show no signs of tetanus intoxication due to lethal toxin.

APPENDIX T.12

IDENTITY TEST

The identity tests on a batch of toxoid can be carried out by both "in vitro" and "in vivo" methods. Although it is a simple procedure to establish the identity of fluid toxoid by a flocculation test, in the case of adsorbed toxoids it is necessary to dissolve the adsorbed material by the addition of sodium citrate.

Appendix T.12

For fluid toxoids a tetanus antitoxin is diluted so as to contain 20 IU per ml. Increasing volumes of 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 ml are pipetted into a series of tubes, the volumes are made up with normal saline to 1 ml and 1 ml of fluid toxoid, diluted to approximately 10 Lf/ml, is added to each of the tubes. The tubes are placed in a water bath at 50°C and observed continuously. The tube containing 0.5 ml of antitoxin or the tube on either side of it may show flocculation and such flocculation establishes the identity of the product.

For an adsorbed toxoid about 0.5 g of sodium citrate is added to 10 ml of the adsorbed toxoid. The mixture is incubated at 37°C for one or two days to dissolve. After the aluminium salt has dissolved the flocculation test similar to one stated above is used. The flocculation occurs in the same way though it may take a longer time.

In the "in vivo" test a suitable dose of toxoid is injected into guinea-pigs and the presence of antitoxin in the guinea-pigs' blood can be demonstrated by the protection test carried out in mice.

APPENDIX T.13

POTENCY TEST

Although the WHO Requirements do not specify any particular method it is essential that potency be measured against that of a national standard which has been calibrated against the International Standard for tetanus toxoid.

The inclusion of a reference preparation is essential because the antitoxin response of mice or guinea-pigs to a tetanus toxoid may be affected markedly by diet, conditions of maintenance, strain of animal used; such variables make it difficult to identify real differences between toxoids.

Appendix T.13

Tests based upon the use of a standard preparation generally involve vaccination of groups of animals with multiple dilutions of the test and reference vaccine, and comparison of the log dose response curves of the preparations. If the response curves are reasonably linear and do not differ significantly in slope (b value) the difference in log dose required to achieve a given response can be calculated to give an estimate of the relative potency of the test preparation, i.e. the ratio of the volume of the standard preparation required to elicit a given response over the value of the test preparation required to elicit the same response. Since the potency of the national or laboratory standard will have been calibrated in International Units, it is possible to assign a potency to the test preparation also in International Units.

1. Suggested method of assay for adsorbed vaccine

(a) In guinea-pigs

Prepare three dilutions in saline of the national or laboratory standard preparation of tetanus vaccine (adsorbed) or of the International Standard, and three similar dilutions of each of the toxoids or vaccines under test. The choice of dilutions used will vary depending upon the strain of guinea-pig and the conditions of maintenance, but successive dilution of each preparation should conform to a twofold series (e.g. 100, 200, 400). The particular dilutions used are selected in the light of experience of local conditions such that the median dose for each preparation tested may be expected to protect approximately half the animals against challenge.

Inoculate 1 ml of the diluted vaccine subcutaneously into each group of guinea-pigs which should contain not less than 16 animals for the subcutaneous lethal challenge. Five unincolated guinea-pigs are used as controls.

Appendix T.13

It is advisable to distribute the guinea-pigs in each treatment group randomly through several cages since the level of response can be affected by local conditions within different cages. When randomization is carried out it is necessary to identify each animal individually. A scheme for the identification of animals by dye marking is shown at the end of this Appendix.

The responses of the guinea-pigs to the single dose of vaccine are determined by challenge 28 days after inoculation. The challenge is given by the subcutaneous injection of 1 ml of a dilution of tetanus toxin containing approximately 100 LD_{50}. A suitable toxin should contain at least 10 000 LD_{50}[1] per Lf. The guinea-pigs are observed twice daily for five days, and the number of animals protected by each vaccine dilution over that period recorded.

The control group of unvaccinated animals is divided into three sub-groups each of two animals which is inoculated with 1 ml of a 1:50, 1:100 and 1:200 dilution respectively of the toxin solution used to challenge the vaccinated animals.

The best method of estimating the relative potencies of the preparations from the proportion of animals surviving the challenge is by parallel line probit analysis (Finney)[2].

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[1] To determine the LD_{50} of a toxin prepare dilutions of the toxin containing 0.0002 Lf, 0.0001 Lf, 0.00005 Lf, 0.000025 Lf per ml, and inject 1 ml doses of each dilution into groups of five guinea-pigs. Observe the animals for four days and count the proportion of animals which die showing symptoms of tetanus during that period. From the data derive the LD_{50} by the method of Reed & Muench.

[2] Probit Analysis, 3rd ed. (Cambr. Univ. Press, London).

Appendix T.13

Since this method is impracticable without a suitable calculator[1] it is suggested that the following simplified methods may be more practicable although they make less effective use of the data:

Reed & Muench, Am.J. Hyg., 1938, 37, 493

Wilson & Worcester, Proc. Nat. Acad. Sci., 1943, 29, 207, or by plotting the percentage protection against $\log_{10}$ dose either on "probability paper" or after conversion with probit tables on ordinary graph paper, and measuring the distance between the regression lines drawn for the test and standard preparations. This distance represents the $\log_{10}$ of the relative potency of the test preparation in terms of the reference.

A test is valid provided that:

(i) all of the control guinea-pigs challenged with 1 in 50 dilution of the challenge dose of toxin die, and that none of the guinea-pigs inoculated with the 1/200 dilution of the challenge dies within the period of observation of the test;

(ii) the lowest dilution of vaccine protects more than half of the animals;

(iii) the highest dilution has protected less than half; and

(iv) that the dose response curves of the test and reference vaccine do not deviate significantly from parallelism and linearity. If no standardized test for parallelism is available a test may be accepted provided that the slope of the steeper curve is not more than 150% that of the shallower curve.

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[1] Probit analysis can be performed with an inexpensive pocket calculator with suitably programmed magnetic cards.

Appendix T.13

In the WHO Requirements no minimum potency for adsorbed tetanus toxoid has been formulated. As a guideline these vaccines should not contain less than 40 I.U./single human dose.

(b) In mice

Prepare three dilutions in saline of the national standard preparation of tetanus vaccine (adsorbed) or of the International Standard, and three similar dilutions of each of the toxoids or vaccines under test. The choice of dilution used will vary depending upon the strain of mice and the condition of maintenance, but successive dilutions of each preparation should conform to a twofold series (e.g. 30, 60, 120). The particular dilutions used are selected in the light of experience of local conditions such that the median dose for each preparation tested may be expected to protect approximately half of the animals against challenge.

Inoculate 0.5 ml of the diluted vaccine subcutaneously into each group of mice which should contain not less than 16 animals for the subcutaneous lethal challenge. Twelve uninoculated mice are used as controls.

The responses of the mice to the single dose of vaccine are determined by challenge 28 days after inoculation. The challenge is given by the subcutaneous inoculation of 0.5 ml[1] of a dilution of tetanus toxin containing approximately 200 LD_{50} per ml.

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[1] A suitable toxin should contain at least 10 000 LD_{50} per Lf. To determine the LD_{50} of a toxin prepare dilutions of toxin containing 0.0002 Lf, 0.0001 Lf, 0.00005 Lf, 0.000025 Lf per ml, and inject 1 ml doses of each dilution into groups of five mice. Observe the animals for four days and count the proportion of animals which die of tetanus symptoms during that period. From this data derive the LD_{50} by the method of Reed & Muench.

Appendix T.13

The mice are observed twice daily for five days, and the number fo animals protected by each vaccine dilution over that period recorded.

The control group of unvaccinated animals is divided into three sub-groups each of four animals which are inoculated with 1 ml each of 1:50, 1:100 and 1:200 dilutions respectively of the toxin solution used to challenge the vaccinated animals.

The best method of estimating the relative potencies of the preparations from the proportion of animals surviving the challenge is by parallel probit analysis (Finney). Since this method is impracticable without a suitable calculator[1] it is suggested that the following simplified methods may be more practicable although they make less effective use of the data:

Reed & Muench, Am.J.Hyg., 1938, 27, 493

Wilson & Worcester, Proc. Nat. Acad. Sci., 1943, 29, 207,

or by plotting the percentage protection against log dose either on "probability paper" or after conversion with probit tables on ordinary graph paper, and measuring the distance between the regression lines drawn for the test and standard preparations. This distance represents the $\log_{10}$ of the relative potency of the test preparation in terms of the reference.

A test is valid provided that:

(i) all of the control mice challenged with 1 in 50 dilution of the challenge dose of toxin die, and that none of the mice inoculated with the 1/200 dilution of the challenge dies within the period of observation of the test;

(ii) the lowest dilution of vaccine protects more than half of the animals;

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[1] Probit analysis can be performed with an inexpensive pocket calculator with suitably programmed magentic cards.

(iii) the highest dilution has protected less than half; and

(iv) the dose response curves of the test and reference vaccines do not deviate significantly from parallelism and linearity. If no standardized test for parallelism is available a test may be accepted provided that the slope of the steeper curve is not more than 150% that of the shallower curve.

It has been found that the presence of pertussis vaccine has a strong adjuvant effect on the tetanus potency in mice. It will be found, therefore, that DPT vaccines show a higher tetanus potency of about 50% when tested in mice than when tested in guinea-pigs. For this reason in some requirements a higher tetanus potency is required for DPT vaccines, when tested in mice. A reasonable potency level for adsorbed tetanus toxoids can be considered to be 40 I.U./single human dose (60 I.U./ single human dose in DPT vaccines when tested in mice).

2. Method of assay for unadsorbed vaccine

The principles for assaying unadsorbed vaccine are similar to those already described for the assay of adsorbed vaccine. There are however four major differences:

(i) the test must be performed in guinea-pigs;

(ii) vaccines are tested against an unadsorbed standard;

(iii) the dose response curve is less steep as a result of which the interval between doses should be threefold rather than twofold;

(iv) the precision of the assay is lower necessitating the use of larger numbers of animals in the experimental groups.

Appendix T.13

3. Suggested method for individual identification of guinea-pigs in an animal room

The following scheme allows for the individual identification of more than 800 guinea-pigs. The identification consists of dye marks on head, back and legs. For recording purposes head and back colours are designated as a capital letter, thus:

A	Red head	R	Mauve head, green back
B	Red back	S	Red head, red back
C	Green head	T	Green head, green back
D	Green back	W	Mauve head, mauve back
E	Mauve head	X	Yellow head
F	Mauve back	Z	Yellow back
H	Red head, green back	AA	Yellow head, red back
K	Red head, mauve back	BB	Yellow head, green back
L	Green head, red back	CC	Yellow head, mauve back
N	Green head, mauve back	DD	Red head, yellow back
P	Mauve head, red back	EE	Green head, yellow back
		FF	Mauve head, yellow back

Leg colours are designated by small letters, and number thus:

Colour	Letter	1	2	3	4	5
red	a	left front	right front	left rear	right rear	both front
red	b	left front +left rear	left front +right rear	right front +left rear	right front +right rear	both rear
green	c	left front	right front	left rear	right rear	both front

Appendix T.13

Colour	Letter					
green	d	left front +left rear	left front +right rear	right front +left rear	right front +right rear	both rear
mauve	e	left front	right front	left rear	right rear	both front
mauve	f	left front +left rear	left front +right rear	right front +left rear	right front +right rear	both rear
yellow	h	left front	right front	left rear	right rear	both front
yellow	k	left front	left front	right front	right front	both rear

Example: Hk4 = Red head, green back, right front and right rear legs coloured yellow

Dyes: Mauve = methyl violet 1%

Green = malachite green 1%

Red = basic fuchsin 1%

Boil 1 litre distilled water. Remove from heat, sprinkle 10 g of dye on the surface and stir in.

Yellow - Picric acid 0.5%

Boil 1 litre distilled water. Remove from heat, sprinkle 5 g of dye on the surface. Do not stir.

The adhesion of dyes to animal furs is improved by the addition of 1 drop Lissopol (BDH) to 100 ml of solution.

Appendix T.13

4. Determination of LD_{50} by the method of Reed & Muench[1]

Example:

Suppose 20 animals per dilution are injected with doses of (expressed in microlitres of vaccine injected): 200, 100, 50 25. The animals are challenged with toxin.

Dose	Animals	Surviving	Σ	Dead	Σ	Perc.mortality
200	20	18	39	2	2	2/41 = 5%
100	20	13	21	7	9	9/30 = 30%
50	19*	7	8	12	21	21/29 = 72%
25	20	1	1	19	40	40/41 = 98%

*Suppose one animal died intercurrently before challenge.

The 50% mortality level is found between doses 100 and 50.

$$\text{Proportional distance} = \frac{50-30}{72-30} = \frac{20}{42} = 0.48$$

Dose just below 50% mortality is 50.

log 50 = 1.699

0.40 x log 2 = 0.144 (0.48 = prop.dist.; 2 = dil. factor)

1.843 +

[1] Amer. J. Hyg. (1938), 27, 493.

Appendix T.13

Antilog = 70 = ED_{50} vaccine

Suppose ED_{50} standard in same test = 40 and standard contains 100 IU/ml

Vaccine contains $\frac{40}{70} \times 100 = 57$ IU/ml

(Calculation can be further simplified by methods of Stanić or Tint and Gillen, Path. Microbiol., 26, 298 (1963); J. appl. Bact., 24, 83 (1961).)

APPENDIX T.14

TEST FOR SPECIFIC TOXICITY

The test for specific toxicity shall be carried out as specified in the Requirements for Biological Substances No.10 (Requirements for Diphtheria Toxoid and Tetanus Toxoid) see Appendix T.27.

For bulk toxoid - Part A, section 3.4.3, page 45.

For final lot - Part A, section 3.5.4, page 46.

APPENDIX T.15

DETERMINATION OF TOTAL NITROGEN (TN) AND NON-DIALYSABLE PROTEIN NITROGEN (PN)

The nitrogen content of samples can be estimated by the following method:

Estimations performed on the sample as received yield total nitrogen (TN) values.

Estimations performed on samples after exhaustive overnight dialysis against running water yield non-dialysable nitrogen (PN) values.

(a) Reagents:

(i) Indicator

1. Methyl red-methylene blue.

   2 parts of 0.2% methyl red are mixed with 1 part of 0.2% methylene blue, both in 95% ethanol.

or 2. Methyl red-brom cresol green.

   5 parts of 0.2% brom cresol green are mixed with 1 part of 0.2% methyl red, both in 95% ethanol.

(ii) Sodium hydroxide-sodium thiosulfate solution - aqueous solution of 50 g of NaOH and 5 g of $Na_2S_2O_3 \cdot 5H_2O$/100 ml.

(iii) Boric acid solution. 4 g of H_3BO_3 is dissolved in 100 ml distilled water.

Appendix T.15

(b) Determination:

A 1 ml sample of dialysed toxoid is measured into a 30 ml Kjeldahl digestion flask. To the sample 1.30 ± 0.05 g potassium sulfate, 40 ± 5 mg mercuric oxide and 2.0 ml concentrated sulfuric acid are added. Porcelain chips or glass beads are added and the mixture is digested for four hours under vigorous boiling with the acid condensing well up into the neck of the flask. The flask is cooled, a minimum of distilled water (about 5 ml) to dissolve the solids is added and a thin film of vaseline is applied to the mouth of the flask. The digest is transferred together with the porcelain chips to a micro or semi-micro steam distillation apparatus of standard design. To condition the apparatus after periods of disuse steam is passed through it for several minutes. Completeness of transfer of the digestion mixture can be tested by the addition of a drop of methyl orange indicator to the final rinse. The 125 ml Erlenmeyer receiver containing 5 ml of 4% boric acid and four drops of the indicator is placed under the condenser with the tip extending below the surface. A volume of 8 ml of the sodium hydroxide-sodium thiosulfate reagent is added to the still and steam distillation is continued until about 15 ml of distillate is collected. The contents of the receiver are diluted to 50 ml with distilled water and the ammonium hydroxide is titrated with 0.02 N HCl. As the end-point the grey colour is chosen or the first appearance of the red one. The blank value is determined using the same amount of reagents rinsing the digestion flask in the same manner and using the same digestion period as for the determination.

It is possible to determine PN also by precipitating the proteins with 5% trichloracetic acid, washing the precipitate and determining TN in it. In this case the obtained value is not "non-dialysable nitrogen", but "TCA-precipitable nitrogen", but both values are very close to "protein nitrogen".

APPENDIX T.16

TEST FOR THIOMERSAL CONTENT

By chemical means

Procedure

The thiomersal content (merthiolate, thimerosal, ethylmercuri-thiosalicylate) is determined spectrophotometrically using diphenylthiocarbazone (dithizone).

Separatory funnels are washed with concentrated nitric acid and rinsed with tap and distilled water. Two aliquots of a standard 0.01% acueous solution of thiomersal (0.5 and 1.0 ml) and two samples of the test solution (1 ml) are added to individual separatory funnels and the volume adjusted to 10 ml with a 1% solution of ammonium acetate at pH 6.0. Ten millilitres of a 1 in 10 dilution of a fresh solution of 0.01% dithizone in chloroform is added to each funnel and the contents shaken vigorously for 45 seconds. The chloroform layer is carefully separated.

The 0 and 100% transmission points on the spectrophotometer are set at 490 nm using the diluted dithizone solution and a scan is taken of the test solutions from 470 to 520 nm. The transmission at 520 nm is plotted against the thiomersal concentration of the standards on semi-logarithmic paper and the concentration of thiomersal in the sample determined.

By microbiological means

Nutrient agar is poured to a depth of 4 mm on a suitable rectangular glass plate set in a metal frame (a large Petri-dish may be used but can accommodate only a few samples). It is essential that the plate is absolutely horizontal when the agar is being poured. Circular wells of approximately 8 mm diameter are cut in the agar so that adjacent wells are at least 30 mm apart. The plate is seeded with sufficient *Staphylococcus aureus* organisms to give a confluent growth

Appendix T.16

at 48 hours. Into each well 100 microlitre samples of 1:2.5, 1:5, 1:10, 1:20, 1:40 and 1:80 dilutions of a standard solution of 1 g/litre sodium thiomersalate are randomly dispersed as well as samples of the test vaccines. The location of each preparation is carefully recorded.

The plates are incubated for 48 hours. The diameter of the zones of inhibition, produced by each of the test vaccines, is measured and compared with the zones produced by the reference dilutions of thiomersal. The effective concentration is expressed in terms of the dilution of reference thiomersal solution to which it mostly closely approximates.

Criteria for acceptance

The thiomersal content should be between 0.005 and 0.02%.

APPENDIX T.17

TEST FOR pH

Procedure

A pH meter with glass and calomel reference electrodes is standardized with standard buffer solutions at pH 5.0 and 7.0. The electrodes are rinsed with distilled water, dried and the pH of the test solution recorded.

Criteria for acceptance

pH value should be 7.0 ± 0.3.

APPENDIX T.18

TEST FOR FREE FORMALDEHYDE

Procedure

1 ml of the vaccine or toxoid is diluted with distilled water to 10 ml. 2 ml of the diluted sample is transferred into 50 ml Nessler tube (with about 65 ml capacity) and diluted with distilled water to about 35 ml. 10 ml diluted sulfuric acid (one vol. H_2SO_4 of sp. gr. 1.84: one vol. water) is added and the fluid is diluted with distilled water to the 50 ml mark. Similarly a series of tubes with known amounts of formaldehyde are prepared to serve as formaldehyde standards for comparison, using a 0.01% dilution of Analar solution of formaldehyde (containing not less than 37% of CH_2O) and selecting quantities close to that expected in the sample. To the sample tube and to each of the standard tubes 10 ml of fuchsin-sulfurous acid test solution is added. The mixtures are allowed to stand one hour at room temperature and a colorimetric comparison of the sample tube with the standards is made.

Fuchsin-sulfurous acid test solution

0.2 g of basic fuchsin is dissolved in 120 ml of hot distilled water and the solution allowed to cool. A solution of 2 g of anhydrous sodium sulfite in 20 ml of distilled water is added and then 2 ml of hydrochloric acid. The solution is diluted with distilled water to 200 ml and allowed to stand at least one hour. This solution must be freshly prepared.

Criteria for acceptance

The vaccine shall be shown to have not more than 0.02% free formaldehyde.

APPENDIX T.19

TEST FOR ALUMINIUM CONTENT

Procedure

To duplicate 3-ml samples of the product in boiling tubes are added 1 ml of concentrated sulfuric acid and six drops of concentrated nitric acid. The tubes are heated until dense white fumes evolve. Heating is continued, with dropwise addition of nitric acid, until the contents of the tubes are colourless. The tubes are cooled and 10 ml of distilled water is carefully added. If the solution is cloudy the tubes are boiled until clear. Sodium hydroxide (50% w/v) is added to each tube until a pink-yellow endpoint is obtained with methyl orange indicator. Any precipate forming is dissolved by the addition of dilute sulfuric acid. The contents of the tubes are transferred to separate 250-ml Erlemeyer flasks, the tubes washed out with 25 ml of distilled water and the washings pooled with the contents of the flasks. To each flask are added 25 ml of M/100 disodium ethylenediamine tetra acetate, and 10 ml of acetate buffer (containing 68 g of sodium acetate, 38.5 g of ammonium acetate, 125 ml of glacial acetic acid in a total volume of 500 ml). The flasks are boiled gently for three minutes and 1 ml of pyridylazonaphthol indicator (0.1% solution in 95% ethanol) is added. Each hot solution is titrated with M/100 copper sulfate solution until a purple-brown endpoint is reached. Simultaneously a blank determination is also carried out using distilled water in the digestion procedure instead of sample. Each ml of M/100 $CuSO_4.5H_2O$ = 0.2698 mg Al+++.

Calculation of the results:

$$\text{mg of Al per ml of sample} = \frac{\text{Blank titre-Sample titre}}{\text{Volume of sample (ml)}} \times 0.2698 \times 4.52$$

Criteria for acceptance

The aluminium content in the final bulk product should not exceed 1.25 mg Al per single human dose.

APPENDIX T.20

TEST FOR STERILITY

Tests for sterility shall be carried out by the tests as specified in the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances)[1].

For tests for bulk toxoid - Part A, Section 5.1.1 shall apply.

For tests on final lot - Part, Section 5.1.2 shall apply.

Care must be taken to ensure that the inoculum of toxoid or vaccine does not, in the concentration used in the inoculated medium, decrease the ability of that medium to support the growth of bacteria from small inocula.

APPENDIX T.21

TEST FOR INNOCUITY

The final bulk and final lot shall be tested for inocuity.

One human dose[2] but not more than 1.0 ml is injected intraperitoneally into each of five adult mice weighing 17-22 g and one human dose, but not more than 5.0 ml into each of two guinea-pigs weighing 250-350 g.

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[1] See Appendix T.28.

[2] The human dose is that stated on the label of the preparation to be injected or in the accompanying information leaflet.

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Vaccine is acceptable if none of the animals shows signs of ill-health in the seven days following the inoculation. If one of the animals dies or shows signs of ill-health during the time specified, repeat the test. The substance passes the test if none of the animals in the second group dies or shows signs of ill-health in the time interval specified.

APPENDIX T.22

PREPARATION OF ALUMINIUM PHOSPHATE SUSPENSION

There are two different ways of producing suitable suspensions of aluminium phosphate, starting either from alum or from aluminium chloride.

A. Method starting from alum ($Al_2(SO_4)_3 \cdot K_2SO_4.12H_2O$)

854 g alum (potassium aluminium sulfate 12 aq) is dissolved in six litres of water and filtered, and similarly 685 g of trisodium phosphate ($Na_3PO_4.12H_2O$) are dissolved in six litres of water. The alum solution is held at about 37°C, as it is slightly oversaturated at room temperature. Both solutions are poured at the same time with mixing into 21 litres of water. The precipitate is centrifuged, the sediment resuspended in 13 litres of water and the suspension centrifuged again. The sediment is finally resuspended in a total volume of 8.1 litres in saline, homogenized, pH adjusted with 5 N NaOH to 6.0 and autoclaved for one hour at 120°C. This quantity is sufficient for preparing 66 litres of a vaccine with 3 mg $AlPO_4$/ml or 0.66 mg Al/ml.

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B. Method starting from aluminium chloride

3 kg of pure aluminium chloride ($AlCl_3.6H_2O$) is dissolved in distilled water, and the volume is made up to 30 litres. The solution is then filtered through a soft (K1) filter sheet. Similarly 4.73 kg of pure trisodium phosphate ($Na_3PO_4.12H_2O$) is dissolved in distilled water, made up to 30 litres and also filtered through K1[1] sheets.

To make the phosphate, 150 litres of distilled water is filtered through K1 sheets into a container of 300 litres capacity. The 30 litres of $AlCl_3$ solution is then slowly added and well mixed before adding the Na_3PO_4 with good stirring until a pH of 5.0 is reached (about 27-29 litres are necessary). Finally 30 litres of distilled water are added, the suspension well mixed, and left standing for seven days.

After seven days the clear supernatant is siphoned off and the same volume of sterile distilled water added, the suspension well mixed and again left standing. After seven days the clear supernatant is again siphoned off, distilled water added to bring the volume to 150 litres and then 100 litres of 0.36% NaCl is added. The total volume is now 250 litres.

The suspension of $AlPO_4$ is distributed with continuous mixing in six-litre volumes in 10-litre bottles, steamed for 30 minutes and autoclaved for 40 minutes at 121°C. After two or three days the sterility of the phosphate suspension is tested. This procedure yields bottles each containing six litres of $AlPO_4$ suspension with 6 ml of aluminium phosphate/ml or 1.32 mg of aluminium per ml.

---

[1] K1 paper can be obtained from Seitz or Carlson Ford.

APPENDIX T.23

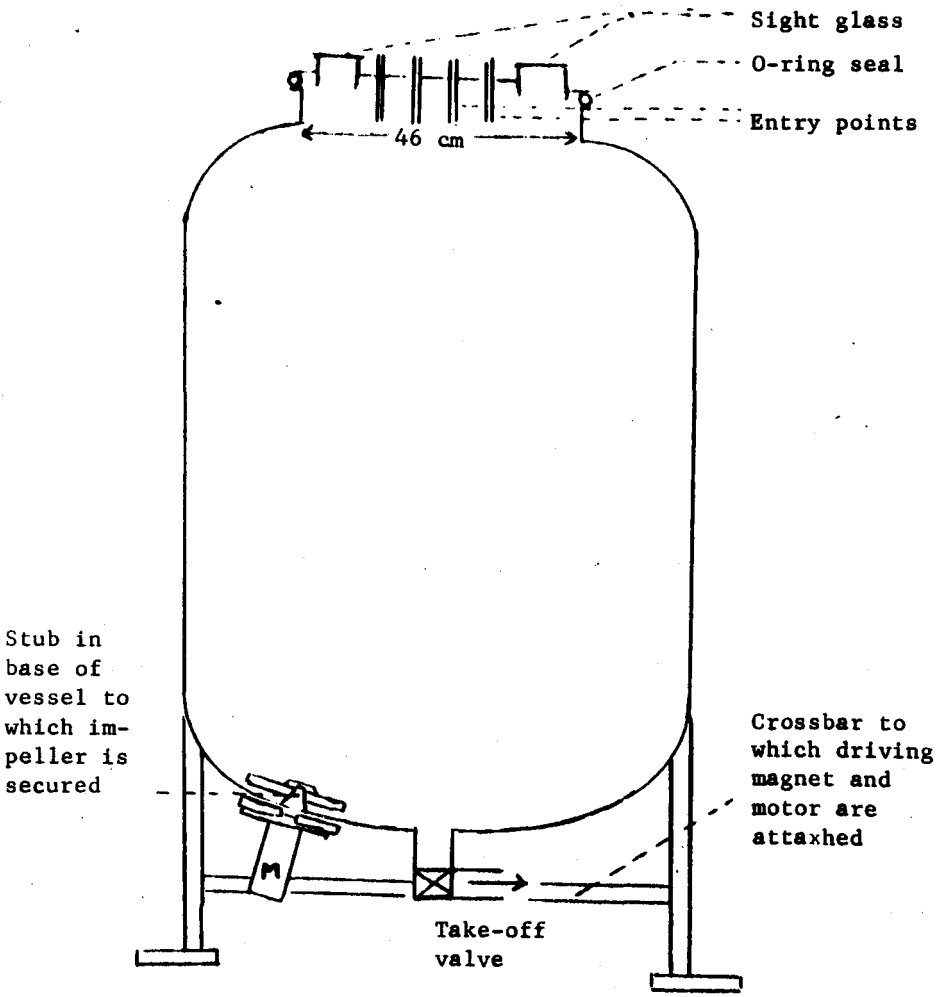
MIXING VESSEL FOR VACCINES
(SEE FIGURE)

Material of construction: stainless-steel, good sanitary quality, e.g. AlS1316, all contact points highly polished. The dimensions are chosen according to the size of the batch which is desirable, and to the size of the autoclave available.

With such simple apparatus magnetic stirring is preferable to ordinary mixers with shafts, propellers, etc., because no special shaft seals, couplings, et. (which often present problems for sterility maintenance) are necessary. The lid is flat, sealed with an O-ring or teflon gasketed and held under pressure by eight or more clamps. At the bottom of the vessel there is an outlet tube. Through the lid there are several inlet tubes. It is advisable to have one separate inlet tube for each component of the vaccine. The outside parts of the inlet and outlet tubes are slightly roughened to improve the connection with rubber, or, preferably, silicon tubing.

Appendix T.23

DIAGRAM OF A SIMPLE MIXING VESSEL



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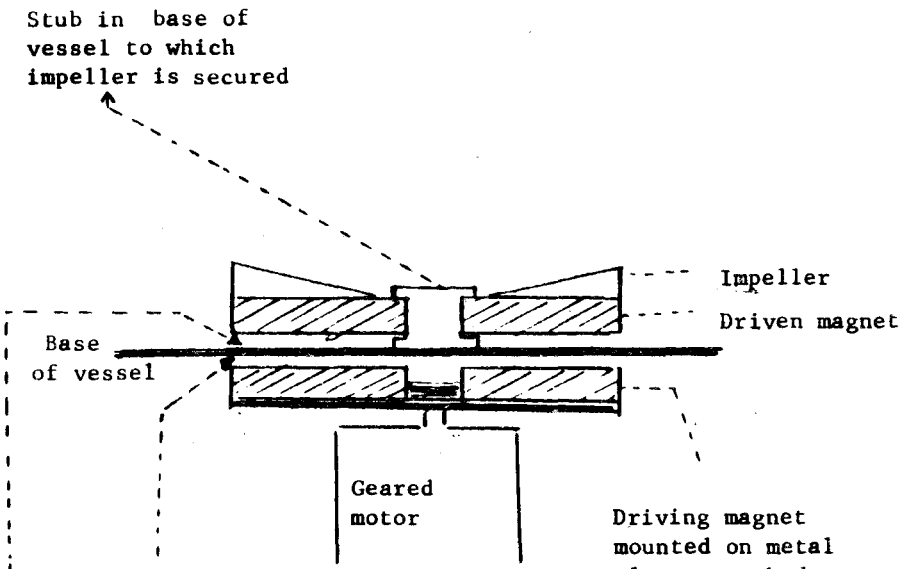
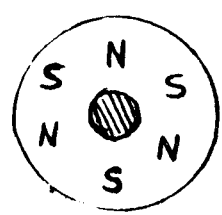


With well-designed units the gap between the magnets may be as much as 4 cm. If the thickness of the vessel is 6 mm, this means gaps between the magnets and the vessel base of up to 17 mm.



Type of multiple ceramic magnet used inside vessel

This equipment is described in detail in Cameron, I. & Godfrey, E. I., Biotechnol. and Bioeng. Symp. (1974) No. 4, 821-835.

APPENDIX T.24

SUGGESTED SOURCES OF EQUIPMENT AND CHEMICALS

The list shown below is representative of manufacturers from whom it is possible to obtain certain equipment, chemicals, etc. It should be stressed that in most cases there are other manufacturers from whom similar materials of equal quality may be obtained. This list, therefore, does not express any preference for the companies mentioned.

The same list is given in the manuals for diphtheria, tetanus and pertussis vaccine production and control. These lists, therefore, are not supplementary. Many pieces of equipment can be used for the production and/or control of different vaccines. Although the production of tetanus toxin requires separate equipment, the bulk toxoid once proven to be sterile and non-toxic, can be further processed in the same area as diphtheria toxoid and pertussis vaccine and the same apparatus can be used.

Equipment

1. Autoclaves for sterilizing under steam pressure. One large autoclave (1.5-2 m^3) (for the fermenter, mixing vessel, etc.) is required, as well as one or two smaller ones (100 - 500 litres). A source of steam with a pressure of about 2.5-3.0 bar is required. If steam is not available then autoclaves with electric steam generators are required.

- AMSCO Co., Erie, Pennsylvania 16512, United States of America
- SUTJESKA, Beograd, Yugoslavia

2. Hot air electric sterilizers, for sterilizing glassware, etc. One large (e.g. height 150 cm, width 80 cm, depth 60 cm internal dimensions) and one small model (e.g. 50 x 50 x 40 cm) are suitable.

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- AMSCO (see 1 above)
- HERAEUS GmbH, Hanau, Federal Republic of Germany
- TIKI, Ljubljana, Yogoslavia

3. Balances. One analytical balance capacity 100 g, sensitivity 0.05 mg. One "technical" balance capacity 1000 g, sensitivity 0.1 g. One "Kitchen" balance capacity 10 kg, sensitivity 5 g.

- E. METTLER, Zurich, Switzerland
- SARTORIUS-WERKE, Göttingen, Federal Republic of Germany

4. pH meter, glass electrode, accuracy $\pm$ 0.05 pH.

- BECKMAN Instruments, Fullerton, CA 92634
  United States of America
- RADIOMETER A/S, Copenhagen, Denmark

5. Jacketed vessel, stainless-steel for preparation of culture media, steam heated, tilting type. Two sizes are convenient, one 120-150 litre and one about 40 litre.

- INOX AG, CH-4600 Olten, Switzerland
- SEITZ WERKE GmbH, 6550 Bad Kreuznach, Federal Republic of Germany

6. Mixing, (blending) vessel for the preparation of final vaccine in bulk.

Manufacturers as under 5 above and :

- CONTACT-ROESTVRIJSTAAL Ltd., Ridderkerk, Netherlands
- T. GIUSTI & SON, 202-228 York Way, Kings Cross, London, England

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7. Container for the filtration of fermenter harvest. Manufacturers are the same as under 5 and 6.

8. Fermenters

- BIOENGINEERING AG, Tannenstr 1, 8630 Rüti ZH, Switzerland
- BIOLAFFITTE, 34 rue de la Muette, 78600 Maisons Laffitte, France
- BIOTEC, Box 11076, 16130 Bromma, Sweden
- R. BRAUN MELSUNGEN AG, 3508 Melsungen, Federal Republic of Germany
- CHEMAP AG, 8708 Männedorf ZH, Switzerland
- CONTACT-ROESTVRIJSTAAL Ltd, Ridderkerk, Netherlands
- ELECTROLUX, POB 69, 31044 Getinge, Sweden
- NEW BRUNSWICK SCIENTIFIC CO., P.O. Box 606, New Brunswick, N.J., United States of America

9. Equipment for filling bulk vaccine into final containers.

- ARENCO GmbH, 7023 Echterdingen, Federal Republic of Germany
- BONAPACE, 20145 Milan, Italy
- STRUERS, Copenhagen, Denmark
- STRUNCK AND CO., 5 Köln 30, Federal Republic of Germany

10. Equipment for washing vials. Manufacturers same as under 9.

11. Vertical laminar flow systems

- GELMAN Instrument SpA, 200090 Opera (Mi), Italy

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- SCHIRP KG, 4711 Bork, Federal Republic of Germany
- THE BABER CO, Sanford, Maine 04073, United States of America

12. Equipment for printing vials

- HAPA AG, Zurich 8, Switzerland

13. Water bath for flocculation reaction. Suitable type is "UNITEMP" Water Bath, Cat. No. 304/1600, Baird & Tatlock, Chadwell Heath, Essex, England.

14. Filtration equipment. It is necessary to have various kinds - one filter press, with about 20 filter plates 20 x 20 cm; one multisheet stainless-steel filter with about 12 plates 20 x 20 cm; three single sheet stainless-steel filters with reservoirs of 10 litres, two litres, 250 ml each.

- BRITISH FILTERS Ltd., Cot Green, Maidenhead, Berkshire SL6 3AG, England
- CARLSON-FORD Ltd., Ashton-under-Lyne, Lancashire, England
- SEITZ Werke GmbH, 6550 Bad Kreuznach, Federal Republic of Germany

15. Membrane filters are very useful for filtration of small volumes of valuable fluids (e.g. concentrated and purified toxoids, etc). One larger filter (diameter about 30 cm), and one smaller (0.14 cm) are necessary.

- MILLIPORE CORPORATION; Bedford, Massachussets 01730, United States of America
- SARTORIUS-MEMBRANFILTER GmbH, Göttingen, Federal Republic of Germany

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16. Metafilter for the filtration of bacterial sludge.

- METAFILTRATION Co. Ltd., Hounslow, Middlesex, England

17. Pure water apparatus. Capacity approximately 100 litre/hour. It is convenient to have demineralized water for washing and general purposes, and pyrogen-free distilled water ("water for injections") for the preparation of injectables. Equipment for demineralized (deionized) water is produced by many manufacturers, e.g.

- ELGA Group, Lane End, Bucks HP14 3JH, England
- MILLIPORE CORPORATION, Bedford, Massachussets 01730, United States of America

High quality distilled water at low cost (no cooling water necessary) by thermocompression principle is produced by:

- STIIMAS SpA, 20090 Settala, Italy

18. Rotary shakers. A small shaker (e.g. for nine Erlenmeyer flasks of one litre capacity) for making subcultures is required. If production of pertussis in shaking flasks is contemplated, then one or several large shakers are required.

- EMENVEE; Gultekdi, Pune, India
- JANKE U. KUNKEL IKA, 7813 Staufen, Federal Republic of Germany
- NEW BRUNSWICK SCIENTIFIC Co., P.O. Box 606, New Brunswick, N.J., United States of America

19. Centrifuge, refrigerated, general purpose, with multiple heads, up to 5000 rpm, with high speed attachment 50 000 rpm. Maximum total capacity about 6 x 1 = 6 litres.

- BECKMAN INSTRUMENTS Inc., Fullerton, CA 92634 United States of America
- MSE Ltd., Crawley, Sussex, England

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20. Centrifuge, continuous flow, is necessary for handling large quantities of the pertussis suspension in fermenter culture. It should be electrically driven, about 20 000 rpm, with three to four one-litre bowls.

- ALFA-LAVAL A.B., Box 1008, S-22103 Lund1, Sweden (Hermetic Clarifier, type BRH 409-34H-11)
- C. PADBERG GmbH, 7630 Lahr, Federal Republic of Germany
- SHARPLES-STOKES Div., Pennwolt Corp., Warminster, Pa. 18974, United States of America

21. Freeze-drying apparatus, small (for drying 20-50 ampoules for maintenance of strains).

- EDWARDS VACUUM Ltd., Manor Royal, Crawley, Sussex, England
- FABRICA INSTRUMENTAL CIENTIFICO, Dr Juan F. Aranguren 76/78, 1405 Buenos Aires, Argentina
- USIFROID, 92 Boulogne-Billancourt, France

22. Fan filter units, to produce a clean atmosphere.

- MICROFLOW, Fleet Mill, Minley Road, Fleet, Hants., England

23. Filter candles for ultrafiltration and filtration of bacterial sludge. At least 200 pieces.

- BERKEFELD FILTER, 3100 Celle, Federal Republic of Germany
- SCHUMACHER FABRIK, Bietigheim, Württ, Federal Republic of Germany

24. Microscope, binocular, with interchangeable eyepieces and objectives, oil immersion.

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25. Spectrophotometer, for general analytical purposes, e.g. Beckman Type ED or similar instrument.

26. Kjeldahl apparatus for nitrogen determination, preferably micro type. Obtainable from any laboratory supply houses.

27. Cold rooms (+ 4°C) and incubator rooms (+ 35°C). It is possible to buy fixed (built-in) or sectional "walk-in" incubators to be assembled on site. The advantages are that they can be enlarged if necessary by adding standard sections.

- HUURRE OY, 33541 Tampere 54, Finland
- LTH, ŠKOFJA LOKA; Yugoslavia
- VOLTAS LTD., Bombay, India (cold rooms + 4°C, - 20°C)

28. Washing equipment for glassware. Generally a simple "bottle brusher" may be adequate.

- THE THOMAS HILL ENGINEERING CO. LTD., Hull, Yorks, England

If more refined equipment is required, then the manufacturers of automatic washers and dryers should be consulted, e.g.

- BETTER BUILT MACHINERY CORP., Saddle Brook, N.J. 07662, United States of America
- METZSCH GmbH, 8264 Waldkraiburg, Federal Republic of Germany

29. Waring or similar type of blender/homogenizer, for homogenization of pertussis suspension. To be obtained from general laboratory supply houses (see Note).

Note: Some of the above-mentioned equipment, as well as all small equipment, glassware, plasticware, etc., can be obtained from general laboratory supply houses such as:

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- ARTHUR H. THOMAS CO., P.O. Box 779, Philadelphia, Pa 19105, United States of America
- BAIRD AND TATLOCK, Freshwater Road, Chadwell Heath, Essex, England
- FISHER SCIENTIFIC CO., 711 Forbes Ave, Pittsburgh, Pa 15219, United States of America
- GALLENKAMP, P.O. Box 290, Technico House, Christopher Street, London EC2P 2ER, England

Chemicals and consumables

30. (Bio) chemicals

- ATLAS CHEMICAL INDUSTRIES Inc., Wilmington 99, Delaware, United States of America (Darco Charcoal)
- THE BRITISH DRUG HOUSES LTD., Poole, England
- CALBIOCHEM AG, 6000 Lucerne 5, Switzerland
- MALLINCKRODT CHEMICAL WORKS, 223 Westside Avenue, P.O. Box 304, Jersey City, NJ 07303, United States of America
- E. MERCK, 61 Darmstadt 2, Federal Republic of Germany
- NORIT SALES CORP. Ltd., Amsterdam, Netherlands (Norit Charcoal)
- SIGMA CHEMICAL CO., P.O. Box 14508, Saint Louis, Missouri 63178, United States of America

31. Bacteriologicals, culture media, etc.: Same firms as above and specialists :

- BENGAL IMMUNITY CO., Calcutta, India (tryptones, casein digests for tetanus medium)
- DIFCO LABORATORIES, Detroit, Michigan 48232, United States of America

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- INSTITUT PASTEUR PRODUCTION, 36 rue du Docteur Roux, 75725 Paris, Cedex 15, France
- OXOID LTD., Wade Road, Basingstoke, Hants RG24 OPW, England
- SHEFFIELD CHEMICAL, 2400 Morris Avenue, Union, New Jersey 07003, United States of America (N-Z-Case TT, N-Z-Amine A)

32. Glassware

- CORNING GLASS WORKS, Corning, NY 14830, United States of America
- JAMES A. JOBLING & CO. LTD., Waltone, Stone, Staffordshire ST15 OBG, England
- JENAER GLASWERK SCHOTT U. GEN., 65 Mainz, Federal Republic of Germany
- VEB JENAER GLASWERK, Jena, German Democratic Republic

33. Filter-paper, filter materials.

- JOHNS MANVILLE, Greenwood Plaza, Denver, Co 80217, United States of America (Hyflo Filter Aid)
- REPUBLIC-SEITZ FILTER CORPORATION, P.O. Box 229, Milldale, Connecticut, United States of America (Republic Filter Papers)
- SCHLEICHER U. SCHÜLL GmbH, 3354 Dassel, Federal Republic of Germany
- WHATMAN INC., 9 Bridewell Place, Clifton, New Jersey, United States of America (Reeve Angel)
- WHATMAN LTD., Springfield Mill, Maidstone, England
- See also manufacturers of filtration equipment, item 14.

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34. Antifoam

- DOW CHEMICAL CO., Indianapolis, Ind. 46260, United States of America
- FARBENFABRIKEN BAYER AG, Leverkusen, Federal Republic of Germany
- HOPKINS AND WILLIAMS, England

35. Rubber and plastic-ware.

- DYNALAB CORPORATION, Box 112, Rochester, NY 14601, United States of America
- NALGE CO., 75 Panorama Creek Drive, Rochester, NY 14625, United States of America
- PHARMA-GUMMI GmbH, 518 Eschweiler, Federal Republic of Germany
- VERNERET, F-94201 Ivry s/Seine, France

APPENDIX T.25

SUMMARY PROTOCOL FOR TETANUS TOXOID PRODUCTION AND TESTING

IDENTIFICATION OF FINAL LOT

Name and address of manufacturer _____

_____

_____

Lot number _____

Date of manufacture of final lot _____

Nature of final product (plain or adsorbed) _____

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Volume of recommended single human dose _____

Number of containers in final lot for each fill volume _____

INFORMATION ON MANUFACTURE

1. Strain

   Identity of Cl. tetani strain used in vaccine _____

2. Single harvest included in final bulk

   Medium used _____

   Period of incubation _____

   Date of earliest harvest included _____

   Conditions of storage _____

3. Bulk toxoid purified/unpurified

   Nature of bulk toxoid _____

   Result of test for antigenic purity if applicable Lf/mg protein N _____

4. Final bulk

   Date of preparation Lf per ml _____

   Result of test for residual free formaldehyde _____

   pH _____

* A list of the identification numbers of the single harvest and bulk purified toxoids should be included.

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5. Adjuvant added

   Nature _____

   mg/Al or Ca _____

6. Preservative

   Nature _____

   Concentration in final product (by assay or calculation) _____

7. Buffer used _____

   Concentration _____

TESTS ON FINAL BULK

1. Sterility

   Date of test and result _____

   Was a repeat test necessary? _____

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INFORMATION ON SAFETY AND POTENCY

2. Specific toxicity test

	bulk purified toxoid	final bulk or final lot
Number of animals		
Date of injection		
Dose of toxoid injected (Lf per animal)		
Route of injection		
Period of test		
Result of test		

3. Potency test

Weight of animals _____

Number and species of animals per dose of toxoid _____

Date of immunization and volume of dilutions administered _____

Date of challenge or bleed _____

Challenge dose of toxin _____

Date of end of test _____

RESULT for challenge test

	Dilution	Survivors / Total	ED_{50}
Reference toxoid I.U./ml			..... ml
Test toxoid			..... ml

Potency of test toxoid _____ I.U. single human dose

95% confidence limits of potency _____

Appendix T.25

TEST ON FINAL LOT

1. Identity test

Test for diphtheria toxoid and result _____

Test for tetanus toxoid and result _____

Test for pertussis and result _____

2. Sterility test

Number of containers examined _____

Method of test _____

Date of start of test _____

Date of end of test _____

Result _____

3. Potency test

If not done on final bulk, report data in space provided for this under final bulk.

4. Innocuity test on final lot	mice	guinea-pigs
Number of animals		
Route of injection		
Volume of injection		
Date of start of test		
Date of end of test		
Result		

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5. Preservative content

Concentration of preservative _____

6. pH

Result of pH test _____

Signature of head of laboratory _____

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

I certify that lot No. of the vaccine satisfies Part A of the WHO Requirements for Tetanus Toxoid.

Signature _____

Name typed: _____

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

APPENDIX T.26

FLOW CHARTS AND DATA RECORDING SHEETS

Introduction

It is useful for a producer of vaccines to make so-called "flow charts" of the whole process. Some examples of such flow charts are shown in this Appendix.

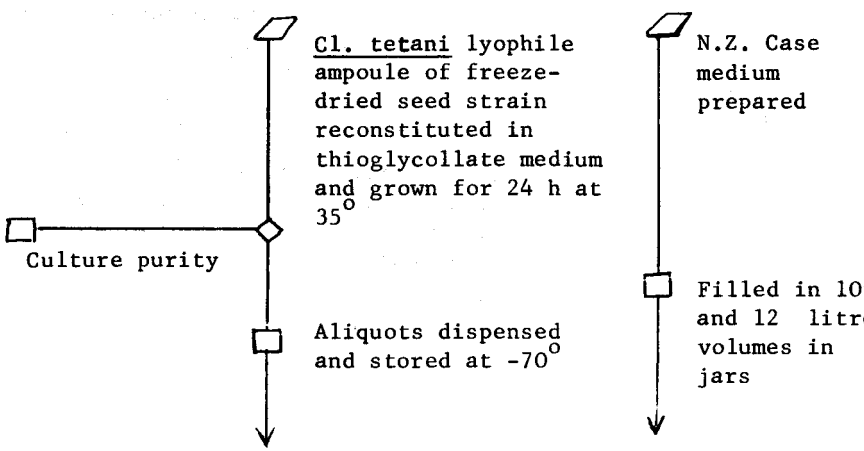
In the text of this manual alternatives have been described for many steps in the production process. The specific flow chart, therefore, will depend on which of the alternatives has been chosen for each individual step in the procedure.

It is useful also for the laboratory workers to record data on work sheets that can be photocopied and accompany the batch of vaccine as it proceeds through the production process. Examples of such data sheets are also included in this Appendix.

PROCESS DESCRIPTION - TETANUS TOXOID (APPENDICES)

EXAMPLE OF FLOW CHART FOR PRODUCTION OF TOXOID

PREPARATION OF CRUDE TOXOID



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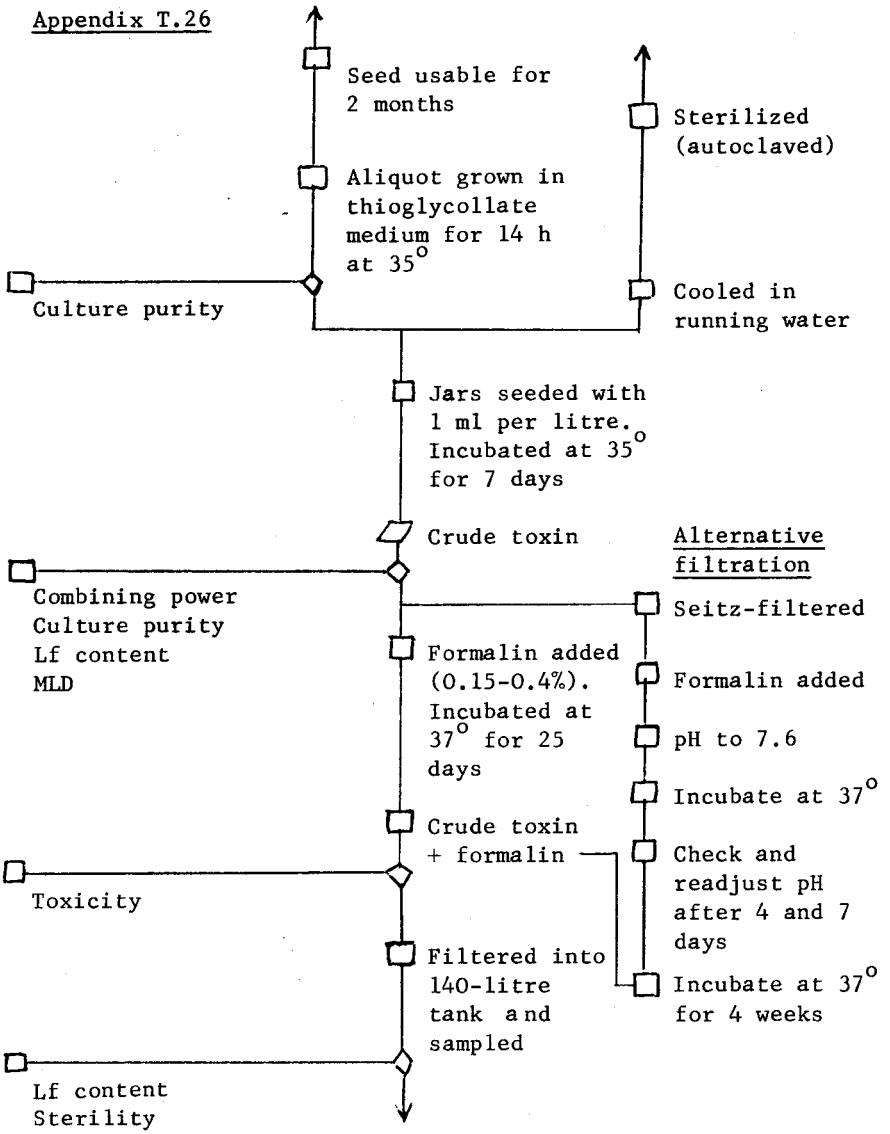

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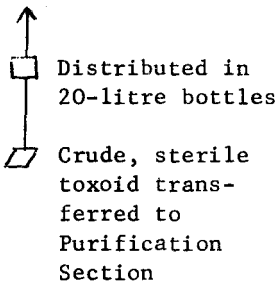
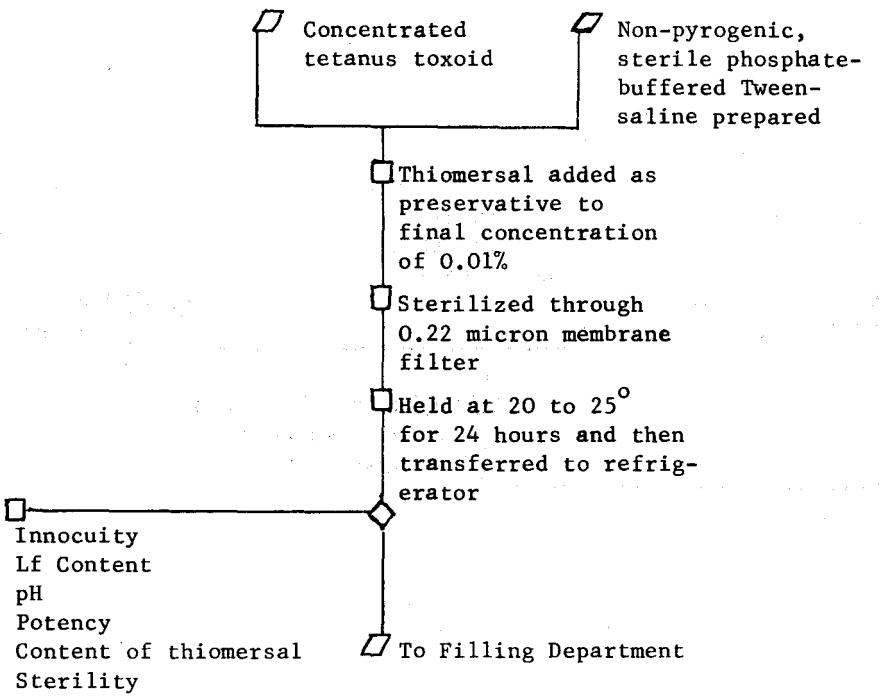


FLOW CHART FOR THE PRODUCTION OF TETANUS TOXOID

PREPARATION OF FINAL PRODUCT - "TETANUS TOXOID (PURIFIED)"



▱ - Input or Output □ - Process stage ◇ - Sampled and held for decision

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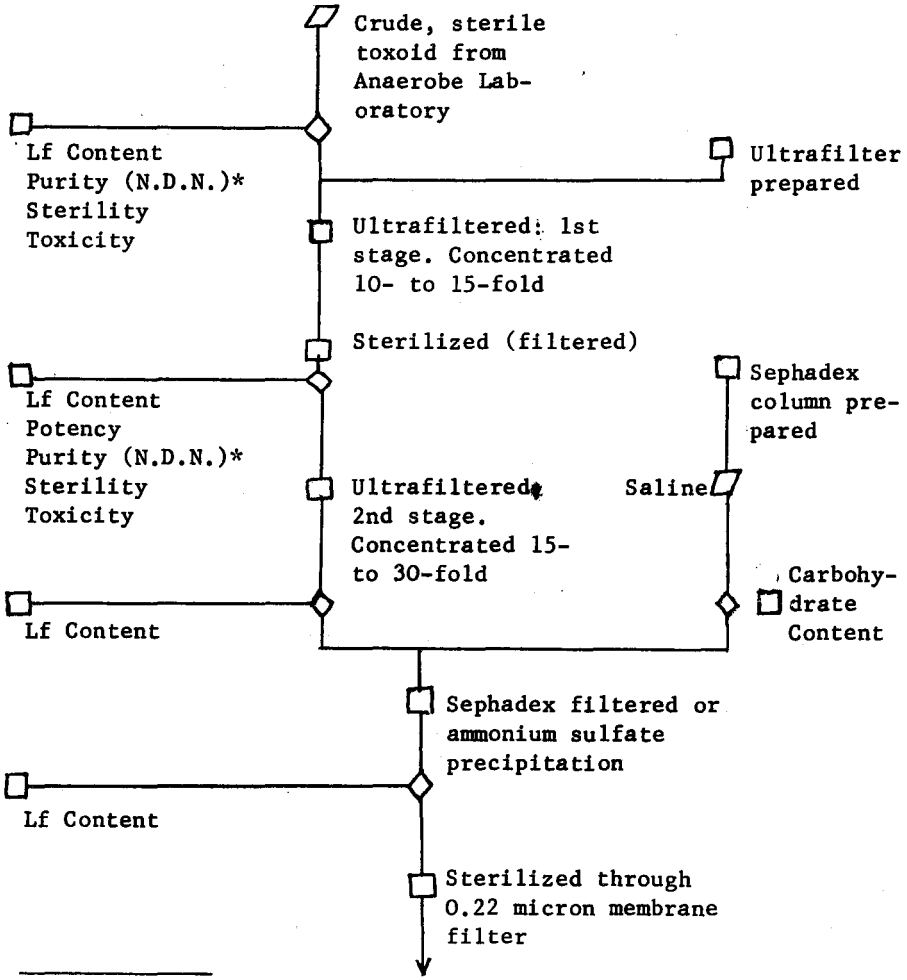
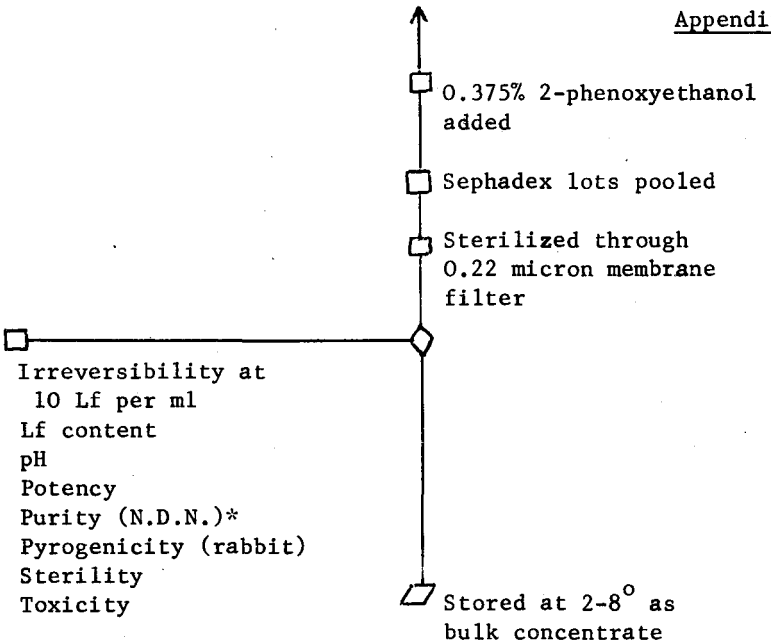
EXAMPLE OF FLOW CHART FOR THE PRODUCTION OF TETANUS TOXOID BY ULTRAFILTRATION

CONCENTRATION AND PURIFICATION OF TOXOID



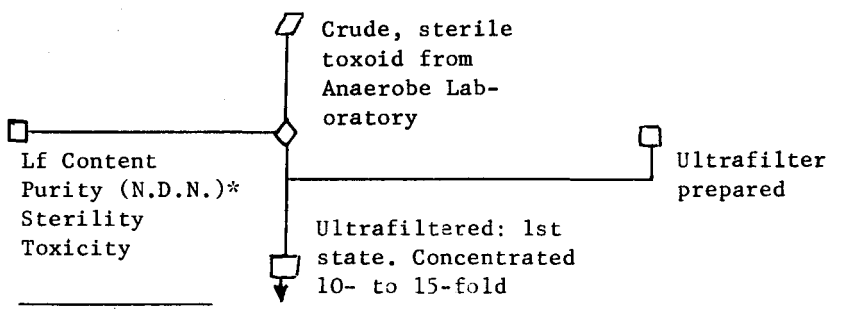
* N.D.N. = Non-dialysable nitrogen.

Appendix T.26



PROCESS DESCRIPTION - TETANUS TOXOID

EXAMPLE OF FLOW CHART FOR PRODUCTION OF TOXOID CONCENTRATION AND PURIFICATION OF TOXOID



*N.D.N. = Non-dialysable nitrogen.

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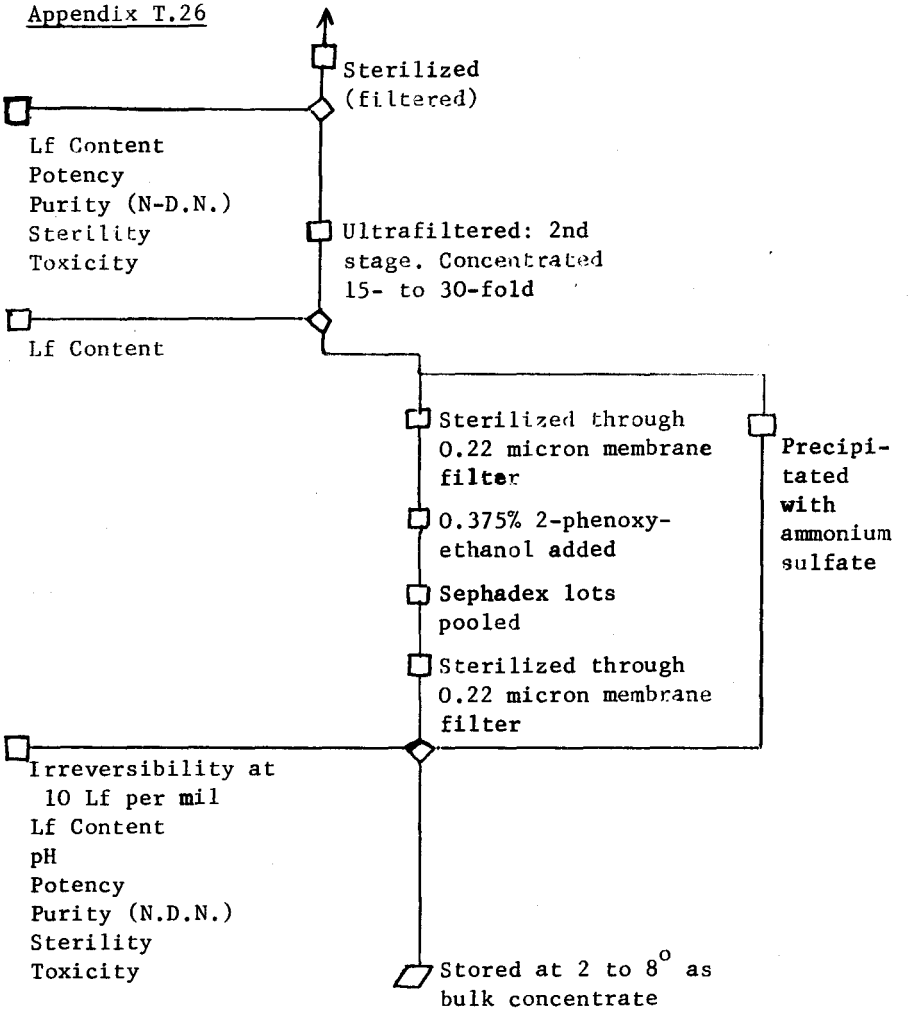

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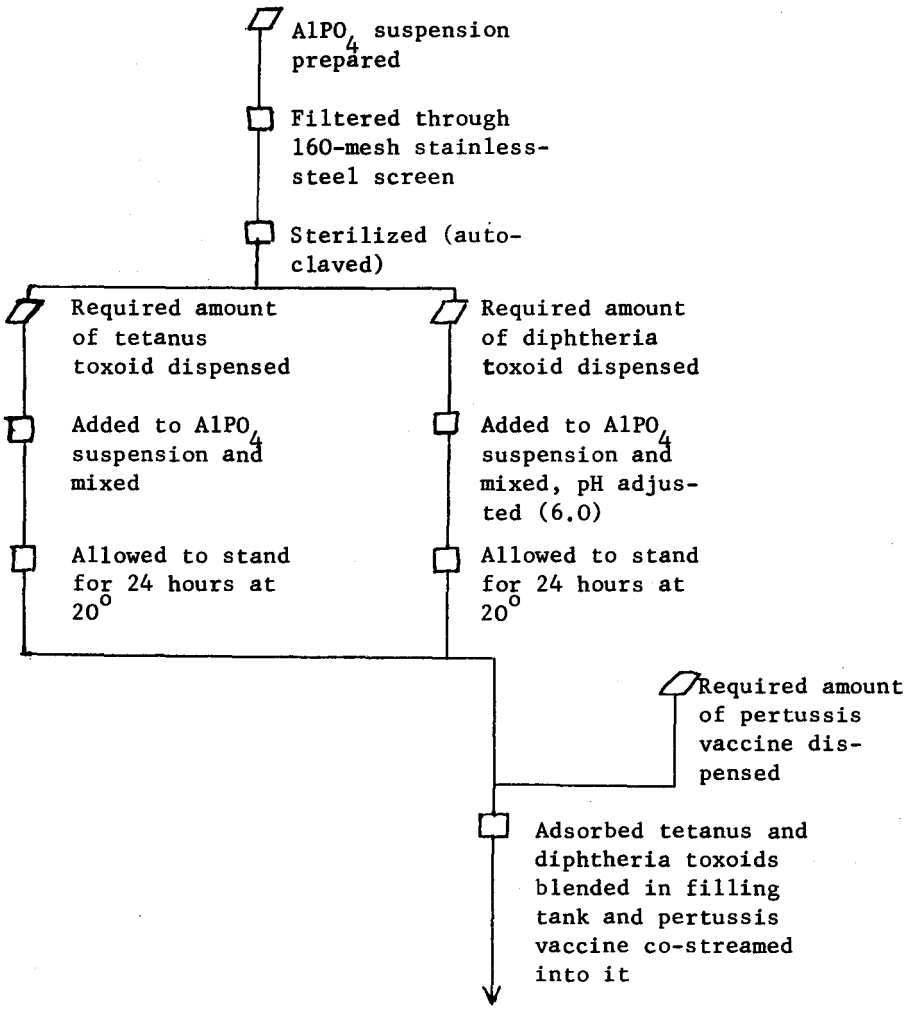
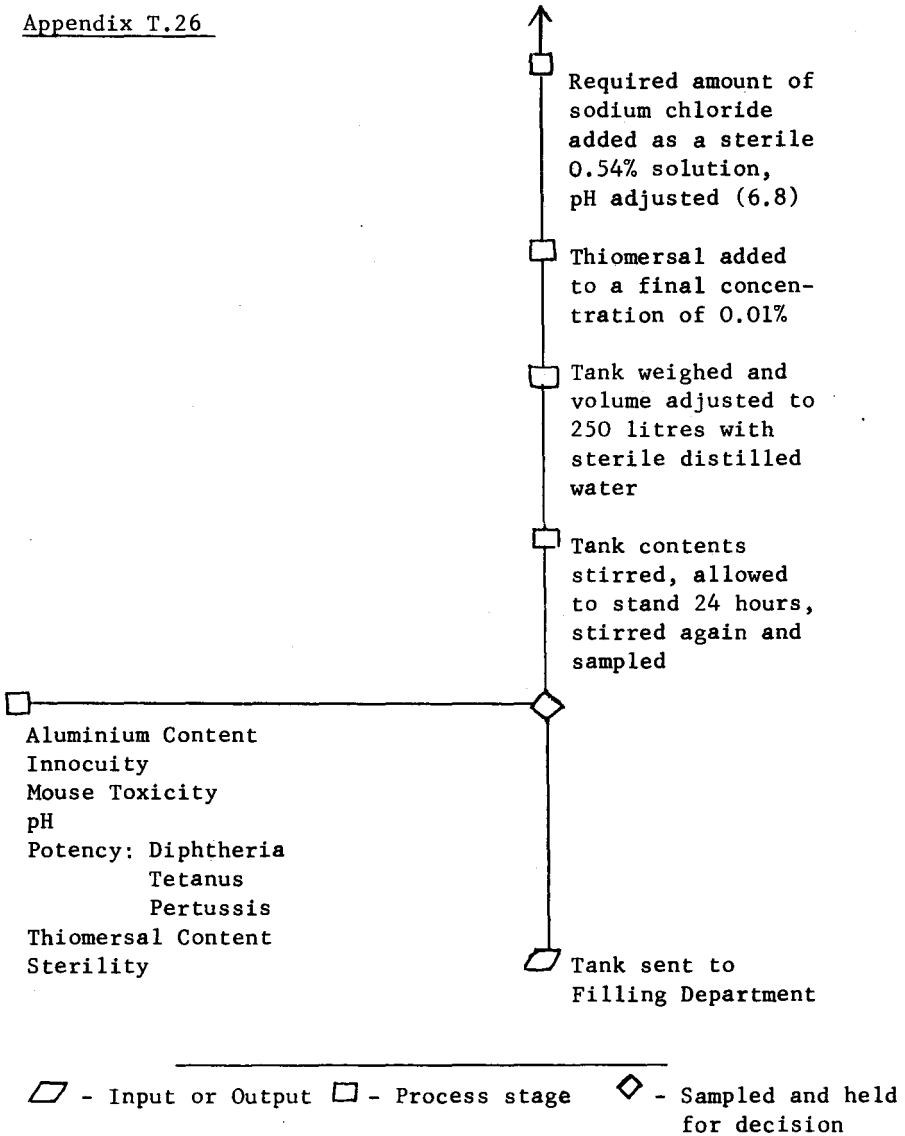
FLOW CHART FOR PRODUCTION OF FINAL PRODUCT



Appendix T.26



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EXAMPLE OF FORM FOR RECORDING MEDIUM PREPARATION

TETANUS MEDIUM

LOT NO._____

DATE_____

PREPARATION OF TRYPTIC CASEIN DIGEST CON ENTRATE

CASEIN DIGEST LOT NO._____ WEIGHT_____g

DISSOLVED IN DISTILLED WATER_____ml

VOLUME MADE UP TO_____ml _____

K_2HPO_4 LOT NO._____ _____g $CaCl_2$ LOT NO._____ _____g

pH ADJUSTED TO_____ WITH _____ml 1ON NaOH

FILTRATION DETAILS_____

_____

PREPARED BY_____

PREPARATION OF FINAL MEDIUM DATE_____

DEXTROSE LOT NO._____ _____g

NaCl LOT NO._____ _____g

$MgSO_4$ $7H_2O$ LOT NO._____ _____g

L-CYSTINE (% SOLUTION)_____ml

SOLUTION III_____ml SOLUTION IV_____ml

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$FeCl_3$ $6H_2O$ (% SOLUTION) _____ml

CASEIN DIGEST SOLUTION
(pH ADJUSTED TO_____ WITH _____ml 5N HCl)____ml

WATER REQUIRED_____ml TOTAL VOLUME_____ml

pH_____

NOTES_____

_____

MEDIA PREPARED BY_____

FINAL VACCINE TETANUS TOXIN LOT NO. (SHEET 1)

MEDIA LOT NO._____ N.Z. CASE LOT NO._____ VOLUME_____ DATE_____

SEED CULTURE DETAILS_____ INIT_____

VOLUME MEDIA	SIZE CONTAINER	NUMBER CONTAINERS	STEAMED (MIN)	AUTO-CLAVED (MIN)	DOWN TIME (MIN)	VOL. SEED/ LITRE	DATE	INIT

CULTURE PURITY_____ INIT_____

POTENCY TESTS ON TOXIN AFTER_____ DAYS GROWTH RATE_____ INIT_____

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LF by FLOCC. & MOUSE TEST

SAMPLE		Units antitoxin							Kf min	Lf	Init
	Flocc.										
	Mouse										
	Flocc.										
	Mouse										
	Flocc.										
	Mouse										

GUINEA-PIG MLD TEST

Toxin dilution	Days to death	MLD	Init

DETOXIFICATION

SAMPLE	VOLUME	FORMALIN VOL %	INIT	TEMP	DATE	DATE REMOVED	REINCUBATION STARTED	REINCUBATION REMOVED	TOTAL DAYS IN INCUBATOR

TOXICITY TESTS

INIT_____

SAMPLE	VOL	NO	G.PIG	DATE	DAYS EXAMINED															
10" JAR																				
12" JAR																				

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(SHEET 2)

TETANUS TOXIN LOT NO._____

FILTRATION DETAILS

DATE OF FILTRATION OF TOXIN PLUS FORMALIN_____

NO. & TYPE CLARIFYING PADS_____

NO. & TYPE STERILIZING PADS_____ INIT_____

STERILITY TEST ON FILTERED TOXOID IN TANK

DATE_____ 10 ml into THIOGLYCOLLATE BROTH_____

10 ml into SOYBEAN-CASEIN DIGEST_____

LOT NO. TOXOID _____

DATE TRANSFERRED TO BOTTLES_____

BOTTLE NO.	VOLUME (L.)
1	
2	
3	
4	
5	
6	
7	
8	

TOTAL VOLUME LOT_____

POTENCY_____Lf/ml

TOTAL Lfs IN LOT_____ml

DATE SENT TO .....

NOTES

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SAMPLE FORM FOR RECORDING DETAILS OF COMBINED VACCINE OF DIPHTHERIA AND TETANUS TOXOIDS AND PERTUSSIS VACCINE ADSORBED

Lot Nos._____
Volume_____litres
Container_____

COMPOSITION

Diphtheria Toxoid: 25(+2) Lf per 0.5 ml
Tetanus Toxoid: 5(+0.5) Lf per 0.5 ml
Pertussis Vaccine: 16 OU per 0.5 ml

Diluent: Aluminium Phosphate Suspension 1.5 mg per 0.5 ml
Preservative: 0.01% Thiomersal

Calculations by:_____

PREPARATION OF VACCINE

A. Vaccine concentrate required

Diphtheria Toxoid: 54 000 Lf per litre x_____litres = _____Lf
Tetanus Toxoid: 11 000 Lf per litre x_____litres = _____Lf
Pertussis Vaccine: 32 000 OU per litre x_____litres = _____OU

Diph.	Lot No.	Lf/ml	Lf used	Volume used	Signature	
					Pooled	Checked
Tet.						
Pert.						

Date:_____

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B. 10% Thiomersal required
1 ml per litre of vaccine = _____ ml

10% Thiomersal added	Lot No._____	_____ml	Sig._____

C. Diluent required

Volume of vaccine	=	_____ ml
Less: Volume of concentrate	=	_____ ml
Less: Volume of preservative	=	_____
Volume of diluent	=	_____ ml

Aluminium Phosphate Susp.	Lot No._____	_____ml	Sig._____
Saline solution		_____ml	Sig._____
Distilled water		_____ml	Sig._____

STIR DURING ADDITION OF COMPONENTS AND BEFORE SAMPLING

SAMPLING OF BULK VACCINE

Test	Volume	Test	Volume
Aluminium content	15 ml	Safety	30 ml
Osmolarity	15 ml	Reference	40 ml
pH and Thiomersal	15 ml		
Potency Dip. & Tet.	30 ml	Sterility	25 ml
Pot.Pert	20 ml	Sterility	25 ml

Date_____
Sig_____
Sig_____

COMMENTS

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REQUIREMENTS FOR TETANUS TOXOID

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 demands for tetanus antitoxin but in the developing world much needs to be done to increase its availability. This is especially the case where tetanus neonatorum 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 Introduction and General Considerations to the Requirements for Biological Substances No. 10.[1] The purpose of these General Considerations is to draw attention to the significant developments that have taken place since those Requirements were formulated.

As with diphtheria toxoid, the most important consideration is the agreement reached on the formulation of requirements for the assay of potency. It has now been accepted that the potency of tetanus toxoid can be measured by an active challenge test and that either guinea-pigs or mice may be used. A comparison has been made of the use of a lethal challenge dose and a paralytic challenge dose and both give similar results when the potency of a test vaccine is compared with a reference preparation. It is interesting to note, however, that when pertussis vaccine is mixed with tetanus toxoid the adjuvant effect of pertussis is more marked in mice than in guinea-pigs. This difference must be recognized when assaying a combined vaccine for the potency of the tetanus component. The minimum acceptable level of potency expressed in International Units, however, must be specified by the national control authority.

Since the last Requirements were formulated in 1964, the International Standard for Tetanus Antitoxin has been

[1] WHO Technical Report Series, No. 293, 1964, Annex 1.

replaced. Furthermore, it has been shown that this second International Standard has an in vivo-in vitro ratio of 1.4 and may be used for the flocculation test assuming that each ampoule contains 1000 Lf equivalents. In addition, an International Standard for Tetanus Toxoid Adsorbed was established in 1965. This was required because the dose/response relationship for adsorbed products was not parallel to that for the plain (non-adsorbed) products. This standard has been most useful in the establishment of national standards required for the quality control of adsorbed products in which tetanus toxoid is a component. It is important that countries should adopt the principle of expressing the potency of tetanus toxoid in International Units rather than Lf which may give misleading information.

These requirements have introduced a test for stability because of the need to ensure that vaccines that may have been subjected to high ambient temperatures will retain their potency.

Although there are few data available to permit the correlation of a potency level in a biological assay with protection in man, and even fewer for the correlation between the potency level and the duration of immunity, sufficient evidence is available to assign a minimum potency level above which a vaccine can be considered to be of an acceptable potency. Such levels have been incorporated into the present Requirements.

In the present International Requirements for Tetanus Toxoid, purification of the product is required. Tetanus toxoid in the unpurified form is liable to give vaccination reactions in man and much work has been done in developing purified material to avoid such reactions. Even with purified products, however, untoward reactions may occur in adults. There is evidence that purification, although enabling more concentrated preparations to be used, may sometimes reduce the immunizing activity of tetanus toxoid, probably on account of the removal of substances having an adjuvant effect. Such purified products can be used

for primary[1] immunization, but it is preferable to combine them either with pertussis vaccine containing whole organisms or with a mineral adjuvant (or both).

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

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning tetanus vaccine, it is recommended that a clause be included which would permit modifications of manufacturing requirements on the condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure that the degree of safety and the potency of the toxoids are at least equal to those provided by the requirements formulated below. It is desirable that the World Health Organization should then be informed of the action taken.

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

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[1] Primary immunization refers to the initial course of injections which usually consists of two (or three) injections at an interval of four to six weeks followed by a further injection seven to twelve months later.

Reinforcing immunity, which is sometimes referred to as "boosting" immunity, refers to subsequent single injections, usually given a number of years later.

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PART A. MANUFACTURING REQUIREMENTS

T.1 DEFINITION

T.1.1 International name and proper name

The international name shall be "Vaccinum tetani". The proper name shall be the equivalent of the international name in the language of the country of origin.

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

T.1.2 Descriptive definition

Vaccinum tetani is a preparation of tetanus toxoid prepared by treating tetanus toxin with formaldehyde. The preparation shall satisfy all the requirements formulated below.

T.1.3 International Standards and International Units[1]

The second International Standard for Tetanus Antitoxin (established in 1969) is stored in ampoules containing dried hyperimmune horse serum containing 1400 IU per ampoule. The International Unit (IU) is defined as the activity contained in 0.03384 mg of the International Standard.

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[1] The expression of the International Unit as a given weight of a dried substance contained in an ampoule is for the purposes of definition only. In practice the International Reference materials should be used by reconstituting the whole contents of the ampoules in a given volume and using the reconstituted material with a calculated unitage per unit volume depending on the volume of fluid used and the declared content of each ampoule.

This standard has an in vivo-in vitro ratio of 1.4 and may be used for the flocculation test assuming that each ampoule contains 1000 Lf equivalents.

The International Standard for Tetanus Toxoid, Plain (established in 1951) is dispensed in the dried form in ampoules containing approximately 833 IU. The International Unit is defined as the activity contained in 0.03 mg of the International Standard.

The International Standard for Tetanus Toxoid Adsorbed (established in 1965) is dispensed in ampoules containing 80 mg of tetanus toxoid adsorbed to aluminium hydroxide, plus an equal part of guinea-pig serum, dried (120 IU per ampoule). The International Unit (IU) is defined as the activity contained in 0.6667 mg of the International Standard.

The above standards are in the custody of the International Laboratory for Biological Standards, Statens Seruminstitut Copenhagen. Samples are distributed free of charge on request to national control laboratories. The international standards are intended for the calibration of national standards for use in the manufacture and laboratory control of tetanus antitoxin and toxoid.

T.1.4 Terminology

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

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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 toxoid 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 working session.

T.2 GENERAL MANUFACTURING REQUIREMENTS

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

All manufacturing processes up to and including the completion of detoxification shall take place in completely separate areas, using separate equipment.

Written descriptions of procedures for the preparation of tetanus toxoid adopted by a manufacturer shall be submitted for approval to the national control authority. Proposals for modifications shall be submitted for approval to the national control authority before their implementation.

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[1] WHO Technical Report Series, No. 323, 1965, Annex 1.

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T.3 PRODUCTION CONTROL

T.3.1 Control of source materials

T.3.1.1 Strains of Clostridium tetani

Strains of Clostridium tetani used in preparing tetanus toxoid shall be identified by a record of their history and of all tests made periodically for verification of strain characters.

A highly toxinogenic strain of Clostridium tetani should be used. A strain that has proved satisfactory in many laboratories is the Harvard strain (No. 49205). Special attention should be paid to maintaining the strain either by freeze-drying or by selective sub-culturing in order to ensure retention of its toxinogenic properties.

T.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 the cultures of the strain from which the parent seed lot was derived. The preparation of the seed lot shall be in compliance with the requirements Part A, section T.3.2.

T.3.1.3 Culture medium for production of toxin

The medium shall be free from ingredients that will be present in the final product and that are known to cause toxic or allergic reactions in man.

Since some medium components may be present in the finished product, it is particularly important to ensure that the final product is free from substances which are likely to cause toxic or

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allergic reactions in man. If the medium is prepared from protein digest, as for example casein hydrolysate or digested muscle, precautions should be taken to ensure that the digestion has proceeded sufficiently to free the medium from such substances. Neither mammalian protein nor human blood group substances should be present in the final vaccine; the method for detecting these substances should be approved by the national control authority.

T.3.2 Production precautions

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

Suitable methods for the production of tetanus toxoid are given in the Manual for the Production and Control of Vaccines: Tetanus Toxoid.[2]

T.3.3 Control of single harvests

The production shall be shown to be consistent by observing the growth, pH and rate of toxin production.

Any culture showing anomalous growth characteristics should be

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[1] WHO Technical Report Series, No. 323, 1965, p. 15.

[2] Manual for the Production and Control of Vaccines: Tetanus Toxoid, WHO/BLG/UNDP/77.2 Rev.1, Section T.7.

investigated and shown to be satisfactory before being accepted as a single harvest.

T.3.3.1 Control of bacterial purity

Samples of the cultures used for preparing a single harvest shall be tested for bacterial purity by microscopic examination of stained smears and 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 preparation.

T.3.3.2 Filtration

After sampling for control of purity the culture shall be filtered using a filter capable of producing a bacteriologically sterile filtrate.

The cultures may be treated with a killing agent (formalin) before filtration. On no account should phenol be used.

If they are not so treated, the culture should be filtered as soon as possible after the end of their incubation period. To facilitate filtration, the cultures may be centrifuged and/or a filter aid added beforehand.

In some countries any filter shedding fibres may not be used.

T.3.3.3 Determination of the concentration of toxin (Lf)

The supernatant of the whole culture prior to inactivation shall be tested by flocculation or other suitable test approved by the national control authority.

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Flocculation is performed in comparison with a reference material calibrated against the second International Standard for Tetanus Antitoxin or an equivalent reference preparation approved by the national control authority.

The time taken for flocculation to occur should be recorded. It has been shown that toxins or toxoids that take an abnormally long time to flocculate are frequently poor in immunizing potency.

It is advisable also to determine the Lf dose together with the minimal lethal dose (mld) for mice.

Suitable methods for these determinations are described in the Manual.[1]

It is preferable that toxic filtrates used in preparing purified toxoid should contain 40 Lf/ml or more.

These tests are not absolute measures of potency but they are a good guide to the consistency of production.

T.3.3.4 Detoxification and purification of toxin

The purification process may precede or follow detoxification and the method used shall be approved by the national control

[1] Manual for the Production and Control of Vaccines: Tetanus Toxoid, WHO/BLG/UNDP/77.2 Rev.1, Appendices T.6 and T.7.

authority. The agent used shall fulfil the requirements of the International Pharmacopoeia[1] or other specifications approved by the national control authority.

Any free formaldehyde shall be removed after the completion of detoxification. The method used shall be approved by the national control authority.

Care should be taken to ensure that reversal to toxin does not take place on storage. A method for the detection of reversal is suggested in the Manual.[2]

In some countries the following test is applied to check the possibility of toxicity reversal. One sample is diluted to 200 Lf/ml (using 0.0167 M phosphate buffered saline), and another sample to a concentration equivalent to that of the final bulk. The latter sample is kept standing at 37°C for 20 days. Of each sample 5 ml are injected subcutaneously into at least five guinea-pigs, which are then observed for 21 days (as described in Part A, Section T.3.4.3).

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

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[1] The Second Edition of the International Pharmacopoeia is "Specifications for the Quality Control of Pharmaceutical Preparations", WHO (1967) p. 228.

[2] Manual for the Production and Control of Vaccines: Tetanus Toxoid, WHO/BLG/UNDP/77.2 Rev. 1, Appendices T.6 and T.7.

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T.3.4 Control of bulk purified toxoid

T.3.4.1 Preparation

The bulk purified toxoid shall be prepared either from a single harvest or a pool of a number of single harvests and shall be sterile.

It is advisable to sterilize the bulk purified toxoid by filtration.

A preservative such as thiomersal may be added to the bulk toxoid.

T.3.4.2 Sterility test

Each bulk toxoid shall be tested for bacterial sterility according to the requirements given in Part A, Section 5, of revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).[1]

T.3.4.3 Specific toxicity test

Each bulk purified toxoid shall be tested for the presence of tetanus toxin by the injection of at least five guinea-pigs, each weighing between 250 and 350 g. Each guinea-pig shall be injected subcutaneously with 1 ml of a dilution of toxoid containing at least 500 Lf of toxoid. Animals that die shall be examined by autopsy. The bulk 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.

T.3.4.4 Test for antigenic purity

Each bulk purified toxoid shall be tested for antigenic purity by determining the Lf value and the concentration of protein

[1] WHO Technical Report Series, No. 530, 1973, p. 48.

(non-dialysable) nitrogen. The Lf determination shall be made using a reference material calibrated against the second International Standard for Tetanus Antitoxin 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 not less than 1000 Lf per mg of protein (non-dialysable) nitrogen.

Measurement of the total combining power (TCP) is also an indication of the quality of the antigen when expressed in relation to the Lf content. A suitable method for measuring the TCP is given in the Manual.[1]

T.3.5 Control of final bulk

T.3.5.1 Preparation

The final bulk material shall be prepared from bulk purified toxoid. The amount of toxoid contained in a single human dose shall be approved by the national control authority and be such that the requirement for potency is fulfilled (see Part A, Section T.3.5.5).

T.3.5.2 Preservative

A suitable preservative shall be added to the final bulk. The preservative used shall have been shown in the amount present in the final bulk to have no deleterious effect on the toxoid or on other vaccine components with which the toxoid may be combined, and to cause no untoward reactions in man. The preservative and its concentration shall be approved by the national control authority.

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[1] Manual for the Production and Control of Vaccines: Tetanus Toxoid, WHO/BLG/UNDP/77.2 Rev.1, Appendix T.10.

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T.3.5.3 Adjuvants

The adjuvant used, its purity and its concentration shall be approved by the national control authority.

Aluminium or calcium compounds are used as mineral carriers. The concentration of aluminium shall not exceed 1.25 mg and that of calcium shall not exceed 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 about half these concentrations are used.

In some countries the adsorbent is precipitated in presence of the toxoid in order to get better adsorption and higher stability.

T.3.5.4 Sterility test

Each final bulk shall be tested for bacterial sterility according to the requirements given in Part A, Section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances, Revised 1973).[1]

T.3.5.5 Specific toxicity test

Each final bulk shall be tested for the presence of tetanus toxin by the injection of at least five guinea-pigs, each weighing between 250 and 350 g. Each guinea-pig shall be injected subcutaneously with a quantity equivalent to at least five single human doses. Animals that die shall be examined by autopsy. The final bulk shall pass the test if no guinea-pig shows symptoms of

[1] WHO Technical Report Series, No. 530, 1973, p. 48.

paralysis or any other signs of tetanus within 21 days of injection and if at least 80% of the animals survive the test period.

T.3.5.6 Potency test

Each final bulk shall be tested for immunizing potency by comparison with a national reference material calibrated against the appropriate international standard. The test shall involve the inoculation of groups of guinea-pigs (weighing 250-350 g) or mice (weighing between 14 and 20 g, but within a 3 g weight range in a single test), and three dilutions of both the final bulk and reference material shall be used. After immunization the animals shall be challenged by a lethal or paralytic challenge dose of toxin given by the subcutaneous route. Standard statistical methods shall be used to calculate the potency of the final bulk. The method adopted and its interpretation shall be approved by the national control authority.

In some countries potency testing is not carried out on each final bulk but on each final lot. In such cases, the provisions of Part A, Section T.5.3, page 48 are applicable.

Sufficient animals should be used to achieve a 95% confidence interval smaller than 50% to 200%.

The details of suitable methods for potency testing are given in the Manual.[1]

The potency of the final bulk shall be approved by the national control authority. The potency of tetanus vaccine containing an adjuvant shall be not less than 40 IU per single human dose. For this purpose the reference material used shall be one calibrated against the International Standard for Tetanus Toxoid, Adsorbed.

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[1] Manual for the Production and Control of Vaccines: Tetanus Toxoid, WHO/BLG/UNDP/77.2 Rev.1, Appendix T.13.

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Tetanus toxoid plain may be used for primary immunization but there is no agreed potency expressed in International Units for this preparation. The potency, therefore, should be approved by the national control authority.

T.3.5.7 Test for residual free formaldehyde

Each final bulk shall be tested for residual free formaldehyde, if this has been used for the detoxification, by a method approved by the national control authority and shall contain not more than 0.02% of residual free formaldehyde.

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

T.3.5.8 Stability test

The stability of the vaccine shall be shown to the satisfaction of the national control authority; at least three consecutive batches of final bulk shall be tested to prove stability during time and conditions of storage. When any changes in the production procedure are made, the vaccine produced by the new method shall be shown to be stable.

The vaccine shall meet the requirements for potency (see Part A, Section T.3.5.6) throughout the dating period at the recommended storage temperature.

Manufacturers should be encouraged to establish the time/temperature relationships of the stability with respect to potency.

T.3.5.9 Test for pH

The pH of the final bulk shall be recorded.

The pH should be between 6.0 and 6.7.

T.4 FILLING AND CONTAINERS

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

Single- and multiple-dose containers may be used. Attention is drawn to the risk associated with the use of multiple-dose containers, and also to the fact that it is inadvisable for the period of time between the withdrawal of the first and the final doses to be unduly prolonged. The filling of an excessive number of doses into multiple-dose containers should therefore be avoided.

It is recommended that a vial once opened should be used on the same day and not reused on subsequent days. If storage and reuse are absolutely unavoidable, the vial must be held in a refrigerator at 5°C±3°C and never for more than one working week.

[1] WHO Technical Report Series, No. 323, 1965, p. 16.

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T.5 CONTROL TESTS ON FINAL PRODUCT

T.5.1 Identity test

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

Flocculation of the toxoid with tetanus antitoxin may serve as an identity test. Tests on toxoid containing an aluminium or calcium carrier may be done after the carrier has been dissolved with a solution of sodium citrate or after the toxoid has been eluted by a suitable method. If the carrier cannot be removed, tests can be made by specific antitoxin neutralization or by antitoxin production in animals.

T.5.2 Sterility test

Each final lot shall be tested for sterility according to the requirements given in Part A, Section 5 of the revised Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances).[1]

T.5.3 Potency test

A potency test shall be made according to Part A, Section T.3.5.6 (p. 46), on each final lot if such a test has not been performed on the final bulk.

T.5.4 Innocuity test

Each final lot shall be tested for abnormal toxicity by the injection of one human dose but not more than 1 ml into each

[1] WHO Technical Report Series, No. 323, 1965, p. 48.

of five mice (weighing 17-22 g) and at least one human dose but not more than 5 ml into each of two guinea-pigs (weighing 250-350 g) by the intraperitoneal route. The tests shall be approved by the national control authority.

The animals shall survive for at least seven days without showing significant signs of toxicity of the product.

T.5.5 Test for adjuvant content

Each final lot of adsorbed toxoid shall be tested for adjuvant content by a method approved by the national control authority (see Part A, Section T.3.5.3), if this test has not been done on the final bulk.

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

T.5.6 Test for preservative content

Each final lot shall be tested for the content of the preservative (see Part A, Section T.3.5.2), if this test has not been done on the final bulk. The test method shall be approved by the national control authority.

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

T.5.7 Test for pH

The pH of each final lot shall be recorded.

The pH should be between 6.0 and 6.7. In some countries this test is applied to the final bulk only.

T.5.8 Inspection of final containers

Each container in each final lot shall be inspected and those showing abnormalities, such as clumping or presence of particles, shall be discarded.

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T.6 RECORDS

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

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

A sample of a suitable summary protocol to be used for tetanus toxoid is included in Appendix T.1 of these requirements.

T.7 SAMPLES

The requirements given in Part A, Section 7, or the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)[2] shall apply.

T.8 LABELLING

The label printed on or affixed to each container and the label on the carton enclosing one or more containers shall show at least:

the words "Vaccinum tetani" and/or the proper name of the product;

the word "Adsorbed" if applicable;

name and address of the manufacturer;

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[1] WHO Technical Report Series, No. 323, 1965, p. 17.

[2] WHO Technical Report Series, No. 323, 1965, p. 18.

the number of the final lot;

the temperature of storage and the expiry date if kept at that temperature;

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 information:

the fact that the toxoid fulfils the requirements of this document;

the nature and amount of preservative present in the toxoid;

the nature and amount of adsorbing agent (if applicable);

the conditions recommended during storage and shipping;

an instruction that the adsorbed vaccine should not be frozen;

instructions for the use of the toxoid and information about contraindications and the reactions that may follow vaccination;

an instruction that the adsorbed vaccine should be shaken before use.

T.9 DISTRIBUTION AND SHIPPING

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

T.10 STORAGE AND EXPIRY DATE

The statements concerning storage temperature and expiry date appearing on the label as required in Part A, Section T.8,

[1] WHO Technical Report Series, No. 323, 1965, p. 18.

shall be based on experimental evidence and shall be submitted for approval to the national control authority.

T.10.1 Storage conditions

The manufacturer shall recommend such conditions of storage and shipping as will ensure that the toxoid conforms to the requirements of potency until the expiry date stated on the label. Adsorbed vaccines shall not be frozen.

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

T.10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory potency test, the date of a potency test being that on which the test animals were first inoculated with the toxoid. When toxoid is issued by the manufacturer, the expiry date shall not be more than three years from the beginning of the potency test.

PART B. NATIONAL CONTROL REQUIREMENTS

T.1 GENERAL

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

The national control authority shall:

Specify the potency requirements;

Approve the method of manufacture;

Approve the method of detoxification and purification;

[1] WHO Technical Report Series, No. 323, 1965, p. 19.

Approve the concentration of preservative and adjuvant;

Approve the test for stability;

Provide the national reference materials.

T.2 RELEASE AND CERTIFICATION

A toxoid shall be released only if it fulfils Part A of the present requirements.

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

The purpose of the certificate is to facilitate the exchange of tetanus toxoid between countries.