U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health
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# MINIMUM REQUIREMENTS: Diphtheria Toxoid

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## 1. THE PRODUCT

1.1 *Definition.* - Diphtheria toxoid is the sterile, aqueous filtrate resulting from the growth of *C. diphtheriae* in a suitable culture medium and which has subsequently been rendered non-toxic as measured by appropriate animal tests but without appreciable loss of its antigenic or combining value. The crude, detoxified filtrate may be refined and the antigen may be prepared in an insoluble form by acceptable methods. Diphtheria toxoid is the proper name of the antigen and in the dissolved form is designated plain diphtheria toxoid (syn.; crude, clear, fluid) in order to distinguish it from the insoluble form which is designated precipitated diphtheria toxoid with a further designation of the precipitant.

1.2 *Culture medium for toxin production.* - Under no circumstances may horse meat or any allergenic derivative from a horse protein be used in the culture medium for toxin production. Likewise, Berna and Witte peptones are excluded from use in the medium because of their pronounced allergenic properties. Preference shall be given to proven non-allergenic ingredients as far as practicable. Consideration shall be given toward avoiding media containing human blood group specific substances. It is recommended that these substances be avoided entirely.

1.3 *Potency of the parent toxin.* - The parent toxin from which diphtheria toxoid is made shall have an L+ dose of not more than 0.20 ml. or an MLD of not more than 0.0025 ml.

## 2. PLAIN DIPHTHERIA TOXOID

2.1 *Formaldehyde for detoxification.* - The formaldehyde used for detoxification is calculated on the basis of a solution of formaldehyde containing not less than 37.0 percent of $CH_2O$. The smallest amount which will bring about complete detoxification as required under section 2.2 and within the generally accepted interval of time shall be used. Since the amount of formaldehyde required is dependent upon the composition of the medium used, it is essential to establish the minimum needed for each medium. In any event the amount originally added must not adversely affect the antigenic quality of the product nor result in a residual of free formaldehyde in the finished product in excess of the permissible amount. This permissible residual must not exceed 0.02 percent of free formaldehyde, $CH_2O$, or 0.05 percent of solution of formaldehyde containing 37.0 percent of formaldehyde.

2.2 *Test for detoxification.* - A volume representing not less than five times the greatest volume intended for any individual human injection, but not less than 2.0 ml., when injected subcutaneously into guinea pigs weighing 300 to 400 grams shall cause no evidence of diphtheria toxin poisoning (extensive necrosis, paralysis, or specific death) at any time during a 30-day period. The animals shall be observed at least daily. No animal dying during the 30-day observation period shall show signs of diphtheria toxin poisoning on necropsy. A sufficient number of test animals shall be injected to insure complete records on at least 4 animals.

2.3 *Test for antigenicity on plain toxoid.* - At least 10 guinea pigs weighing 270 to 320 grams each at the beginning of the test, and sex at random, shall each receive as a subcutaneous injection not more than one-sixth the volume of plain toxoid which is to be recommended for the lot under test as the total human immunizing dose (section 5.2). At the expiration of not more than 6 weeks each of the test animals shall be challenged subcutaneously with not less than 10 MLD of a stable diphtheria toxin contained in a 2 ml. volume. Eighty percent of *all* of the animals challenged (not less than 10 animals) shall survive for at least 10 days. The toxicity of the challenge toxin dilution used shall be confirmed by the injection of 2 ml. of a 1:10 dilution of the challenge dose into each of 2 or more guinea pigs

weighing 250 grams ± 10 percent. Each toxin control guinea pig shall die of diphtheria toxin poisoning within an average time of not more than 4 days. The gross pathology present is principally necrosis or extensive oedema at the site of injection, congestion and injection of the adrenals, and an increased amount of pleural and peritoneal serous exudate.

## 3. *ALUM PRECIPITATED DIPHTHERIA TOXOID*

3.1 *Quantity of alum allowed.* - Alum used for the preparation of alum precipitated diphtheria toxoid shall be not less than 99.5 percent pure potassium alum, Al $K(SO_4)_2.12H_2O$. The finished toxoid shall contain not more than 15 mg. of alum in any individual human injection as determined by analysis of the finished product. In lieu of such analysis the amount shall not be more than a calculated 20 mg. based on the assumption that the total amount added is recovered in the finished product. When aluminum is used in any other form the permissible amount shall not exceed the amount permitted when it is used as potassium alum. In all instances the amount of aluminum used shall be the minimum needed to accomplish the purpose intended.

3.2 *Quality of the parent plain toxoid.* - Precipitated diphtheria toxoid shall be prepared only from plain toxoid which meets all of the minimum requirements for plain toxoid except that it need not necessarily equal the antigenic value required of plain toxoid. Neither is it required that an antigenicity test be made on the parent plain toxoid since this test is subsequently required on the precipitated product before release.

3.3 *Test for antigenicity on precipitated toxoid.* - A volume of precipitated diphtheria toxoid, representing not more than one-half the volume which is to be recommended for the lot under test as the total human immunizing dose (section 5.2), when injected subcutaneously into normal guinea pigs weighing 500 grams ± 10 percent shall produce at least 2 units of antitoxin per milliliter of serum in not more than 4 weeks in an aliquot serum-pool from not less than 4 guinea pigs. Irrespective of the number of guinea pigs used in the test the serum-pool must contain aliquot portions of serum from all animals surviving the immunization period. The unitage of the pooled serum is determined by mixing it with test toxin and injecting the mixture into at least 2 guinea pigs. The L+ dose of the toxin shall be determined against the N. I. H. standard antitoxin in at least 2 guinea pigs.

3.4 *Volume in the final container.* - The final container of precipitated diphtheria toxoid shall contain enough material to deliver not more than 5 complete human immunizing doses, regardless of the volume designated for each injection.

## 4. *ADDITIONAL SPECIFIC TESTS ON THE PRODUCT*

4.1 *Identity test.* - A specific identity test shall be made on the contents of a final container which has been selected at random from each filling of each lot or portion of a lot.

4.2 *Safety test.* - A safety test shall be made on the contents of a final container selected at random from each filling of each lot or portion of a lot. The parenteral injection of the maximum volume tolerated but not more than 0.5 ml. for mice weighing approximately 20 grams each and 5.0 ml. for guinea pigs weighing approximately 350 grams each shall cause neither significant symptoms nor death. At least 2 animals of each species are used and the observation period is not less than 7 days.

4.3 *Sterility test.* - Tests shall be made on final containers selected at random after each filling from each lot. Three containers shall be tested if the total filled is 100 or less and then one additional container for each 50, but not more than 10 final containers need be tested. The volume to be tested shall be the maximum volume recommended for any individual human injection but in no instance less than 1 ml. The culture is made in one or more tubes of fluid thioglycollate medium and incubation is at 35° to 37° C. with observation for not less than 7 days. For additional information refer to the Memorandum of Details.

4.4 *Test for Presence of Blood Group Specific Substances.* - As a control on the method of preparation, a sample of a bulk or finished lot of toxoid shall be tested to insure that the toxoid contains less than 0.004 mgms. of Blood Group A Substance per ml. and less than 0.08 mgms. of Blood Group B Substance per ml. The method of test may be that which is attached to this amendment or any other method which provides similar information. Tests of this type should be made on a series of lots to establish that the method of production insures a product meeting these requirements, but when the method has been established it is not necessary that every lot be tested. Whenever a change is made in the production method it is necessary that the new method be established as satisfactory in this regard. (See Appendix B).

5. *GENERAL REQUIREMENTS*

5.1 *Preservative.* - It is required that the finished product contain a preservative. However, the use of a phenaloid compound is not permitted.

5.2 *Dose.* - The recommended individual human injection shall be not less than 0.5 ml. and not more than 1.0 ml. The total human immunizing dose recommended for plain diphtheria toxoid shall be not less than 3 individual human injections, the total volume of which shall be 6 times the volume used in section 2.3 for determining antigenicity. The interval between injections of the plain toxoid should preferably be 3 to 4 weeks. The total human immunizing dose recommended for precipitated diphtheria toxoid shall be 2 individual human injections, the total volume of which shall be 2 times the volume used in section 3.3 for determining antigenicity. The interval between injections of the precipitated material should preferably be 4 to 6 weeks.

5.3 *Labeling.* - The labeling of plain and precipitated diphtheria toxoid complies with the requirements detailed in the regulations.

5.4 *Dating.* - The expiration date of diphtheria toxoid is 2 years from the date of manufacture or date of issue. The date of manufacture, for dating purposes, is the date of last passing a satisfactory potency test. The date of issue may be 1 year after the date of manufacture provided storage is at not over 5° C. Freezing is not recommended.

5.5 *Storage.* The recommended storage temperature to be stated on the label for the dating period is: "Keep at 2° to 10° C. (35.6° to 50° F.)."

5.6 *Requirements for release.* - Distribution of the product shall be withheld pending a release from the National Institutes of Health. For this purpose the following shall be submitted:

a) A 50 ml. sample which shall be representative of the entire lot.

b) A protocol similar in form to the sample shown in Appendix A and giving the information indicated. A protocol in a trial portion of a lot of either plain or precipitated toxoid shall not constitute valid evidence.

6. *DIPHTHERIA TOXOID IN MULTIPLE ANTIGENS*

6.1 *Permissible combinations.* - A multiple antigen preparation will be accepted for license, as it relates to the diphtheria toxoid component, provided; that the antigenic value of the diphtheria toxoid present in the finished preparation is not less than the total human immunizing dose recommended in section 5.2, that the interval between the first and last injections is not less than the interval recommended in section 5.2, and that the volume of diphtheria toxoid in any individual injection is not more than 1.0 ml. However, the total immunizing dose of toxoid may be divided into more individual injections than are recommended in section 5.2 if the other antigens present make this necessary.

6.2 *Antigenicity test on toxoid in multiple antigens.* - The antigenicity test on diphtheria toxoid in a multiple antigen product may be performed either before or after the introduction of the toxoid into the mixture, except that if the effect of the mixing on the antigenic value of the toxoid is not known, or if it is known to cause a change in the antigenic value, the antigenicity test must be applied after the mixture has been made and adjusted to its final volume.

6.3 *Volume of the multiple antigen injection.* - The volume of the individual human injection will be influenced by its composition. However, the volume shall not be less than 0.5 ml. and whenever feasible not more than 1.0 ml.

6.4 *Volume of the multiple antigen final container.* - Final container of a multiple antigen containing a precipitated antigen shall contain enough material to deliver not more than 5 complete human immunizing doses, regardless of the volume designated for the individual human injection.

6.5 *Dating of the multiple antigen.* - The dating of the multiple antigen and the establishment of the date of manufacture and date of issue is determined by the antigen component having the most restrictive requirements.

6.6 *Requirement for release for multiple antigens.* - Release of multiple antigen products follows the procedure prescribed in section 5.6. The protocol should include:

a) The lot number

b) The date of last satisfactory potency test or National Institutes of Health release

c) The volume of each antigen entering the multiple product

d) The lot number of the resulting multiple antigen

e) Reports of the antigenicity tests mentioned in section 6.2 if these are required by the method of processing or if performed through choice by the manufacturer

f) The number of individual injections in the total immunizing human dose

g) The volume of each injection.

APPENDIX A

Release Protocol for _____ Plain Toxoid

1. Manufacturer: _____ Date: _____ Lot: _____

2. Plain Toxoid Production: _____
Batch numbers

General production items:

a. MLD value per ml.

b. L+ value per ml.

c. Lf value per ml.

d. Percent formaldehyde ($CH_2O$) added

e. Percent residual $CH_2O$ remaining

f. Volume of human injection

3. *Test for Detoxification (Guinea pigs weighing 300-400 gm.):*

a. No. g. pigs used:_____Dose: _____ No. days observed:_____

b. No. g. pigs surviving the full 30-day period: _____

c. No. g. pigs dying from other causes, recorded as survival days: _____

4. *Test for Antigenicity (G pigs weighing 270-320 g.):*

a. No. g. pigs used: _____ Toxoid dose: _____Interval before challenge:_____days

b. Total challenged: _____ Deaths with days survived: _____

c. Percent of all challenged g. pigs surviving:_____

d. No. of challenged g. pigs gaining weight: _____No. losing weight: _____

e. Toxicity of challenge toxin: _____ g. pigs survived: _____ hours

5. Has the lot passed satisfactory tests for: Safety? _____ Identity? _____

Sterility?_____

6. Remarks:

_____
(To be signed by responsible operator)

APPENDIX A

Release Protocol for _____ Precipitated Toxoid

1. Manufacturer: _____ Date: _____ Lot: _____

2. Quality of Parent Plain Toxoid: _____
Batch numbers

General production items:

a. MLD value per ml.
b. L+ value per ml.
c. Lf value per ml.
d. Percent formaldehyde ($CH_2O$) added: _____ Residual $CH_2O$: _____

3. Test for Detoxification (Guinea pigs weighing 300-400 g.):

a. No. g. pigs used: _____ Dose: _____ No. days observed: _____
b. No. g. pigs surviving the full 30-day period: _____
c. No. g. pigs dying from other causes. recorded as survival days: _____

4. Production details of precipitation:
Batch numbers

General production items:

a. Grams of alum per 100 ml. plain toxoid
b. Calculated mg. of alum per dose
c. Assayed mg. of alum per dose
d. No. of washings of precipitate
e. Final vol. percent (AP td/plain td)
f. Volume of human injection

5. Test for Antigenicity (Guinea pigs weighing 500 g. ± 10 percent)

a. No. g. pigs used: _____ AP toxoid doses: _____ Interval before bleeding _____ days
b. Total g. pigs in serum-pool: _____ Total g. pigs surviving immunization: _____
c. Test: _____ ml. pooled serum + _____ ml. or mg. toxin: _____ g.p. survived _____ hrs.
_____ ml. " " + _____ ml. or mg. toxin: _____ " " _____ "
_____ ml. " " + _____ ml. or mg. toxin: _____ " " _____ "
d. CONTROL: 1 unit std. antitoxin + _____ ml. or mg. toxin: _____ g.p. survived _____
e. Units of antitoxin per ml. of pooled serum _____

6. Has the lot passed satisfactory tests for: Safety? _____ Identity? _____

Sterility? _____

REMARKS:

_____
(To be signed by responsible operator)

## APPENDIX B

## METHOD FOR DETERMINATION OF BLOOD GROUP SPECIFIC SUBSTANCES IN TOXOIDS

*Blood Group A Substance*

*1. Toxoid:*

Dilutions are prepared in saline starting at 1:2 and increasing at three-fold increments until an end point is obtained. 0.1 ml. of each dilution is added to each tube.

*2. Control:*

Dilutions of a control preparation* containing 1 mg. of Blood Group A Substance per ml. are prepared in the same manner as the dilutions of toxoid. The Blood group A Substance dilutions should cover the range 200-600-1800-5400.

*3. Anti-A Blood Grouping Serum:*

To each tube containing the toxoid dilutions and the dilutions of Blood Group A Substance is added 0.1 ml. of blood grouping serum diluted to contain two test doses of antibody. One test dose of antibody is defined as the highest of two-fold dilutions of serum which will produce 4+ agglutination when 0.1 ml. of serum dilution and 0.1 ml. of 1% human A cells are mixed and held 15 minutes at room temperature. The degree of agglutination is read after centrifuging the mixtures for one minute at 1500 RPM.

*4. Test:*

The mixtures are held at room temperature for 10 minutes. Then 0.1 ml. of 1% suspension of human A cells is added and the mixtures held 15 minutes at room temperature. The degree of agglutination is read as in the titration of the Anti-A Blood Grouping Serum.

*5. Result:*

The titer of A Substance in the toxoid is recorded as the number of milligrams of A Substance in 1 ml. of toxoid. This is calculated by dividing the highest dilution of toxoid which shows inhibition of hemagglutination with the highest dilution of the control Blood Group A Substance which shows similar inhibition of hemagglutination.

*Blood Group B Substance*

In testing for Blood Group B Substance in toxoid, a similar procedure is followed. In this instance, the dilutions of the control Blood Group B Substance cover the range 9-27-81-243-729 and the appropriate Group B reagents are used.

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* A control preparation of Blood Group Substances A and B may be obtained by making request to the Division of Biologics Standards.