U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
National Institutes of Health
Bethesda 14, Maryland
4th Revision, December 15, 1952

Minimum Requirements: Tetanus Toxoid

TABLE OF CONTENTS

Appendixes

Appendix A.

The mouse test for titration of pooled guinea pig serum for determination of its tetanus antitoxin content.

Appendix B.

Release Protocol for Plain Tetanus toxoid.

Appendix C.

Release Protocol for Tetanus Toxoid, Adsorbed or Precipitated.

Appendix D.

Other assay methods which may be helpful to manufacturers of tetanus toxoid.

1. Method for determining the Lf titer of Tetanus toxoid.
2. Method for determining the total nitrogen of toxoid.
3. A method for the Assay of Free Formaldehyde in Biologics.
4. References to assay procedures found in published articles.

1. *THE PRODUCT*

1.1 *Definition.-* Tetanus toxoid is the sterile aqueous filtrate which is obtained from the growth of *Cl. tetani* in a suitable culture medium and which has been rendered non-toxic, as measured by appropriate animal tests, 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. Tetanus toxoid is the proper name of the product and in the dissolved form is designated plain tetanus toxoid in order to distinguish it from the insoluble form which is designated precipitated or adsorbed tetanus toxoid with a designation of the precipitating or adsorbing agent.

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 should be given toward avoiding media containing human blood group specific substances. (SEE AMENDMENT NO. 1)

1.3 *Potency of the parent toxin.-* The parent toxin from which tetanus toxoid is made shall have (1) a test dose of not greater than 0.01 ml. when tested against 0.1 unit of antitoxin as in the potency testing of tetanus antitoxin, or (2) an MLD for the guinea pig of not greater than 0.0001 ml. A toxin of high test dose is desirable.

2. *PLAIN TETANUS TOXOID*

2.1 *Formaldehyde for detoxification.-* The smallest amount of formaldehyde which will bring about complete detoxification within the generally accepted interval of time shall be used. Since the amount 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 affect adversely the antigenic quality of the product nor result in a residual of free formaldehyde* in the finished product in excess of 0.02 percent expressed as free formaldehyde (HCHO), or 0.05 percent expressed as formaldehyde solution 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 produce no symptoms of tetanus toxin poisoning, as described in section 2.3, at any time during a 21-day period. The animals shall be observed at least daily. A sufficient number of test animals shall be injected to insure complete records on at least 4 animals.

2.3 *Test for potency of plain toxoid.-* The sample used for the official potency test shall be a portion representative of the entire lot taken from

---

* See Appendix D_3

the lot after the completion of all those processes of manufacture which may affect the potency of the final product. At least 10 guinea pigs of either sex, weighing 300 to 400 grams each, at the beginning of the test, shall each receive as a subcutaneous injection an amount of undiluted toxoid of not more than one-third the volume 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 tetanus 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 350 grams ± 10 percent. The toxin control guinea pigs shall die of tetanus toxin poisoning within an average time of not more than 4 days. Symptoms of tetanus toxin poisoning determined by visual inspection and handling are increased muscle tonus, curvature of the spine, asymmetry of body outline when the resting animal is viewed from above, stiffness or paralysis of neck or legs, and inability to rise from a smooth, flat surface when the animal is placed on its side; or any combination of the above.

3. PRECIPITATED OR ADSORBED TETANUS TOXOID

3.1 Quantity of alum allowed.- Alum used for the preparation of alum precipitated tetanus toxoid shall be not less than 99.5 percent potassium alum [Al $K(SO_4)_2 . 12H_2O$.] The finished toxoid shall contain not more than 15 mg. of alum in an individual human injection as determined by analysis of the finished product. In lieu of such analysis, the amount added for precipitation shall be not more than 20 mg. in an individual human injection. When aluminum is used in any other form, the permissible amount shall not exceed the amount permitted as potassium alum. In all instances the amount of aluminum compound used shall be the minimum needed to accomplish the purpose intended.

3.2 Quality of the parent plain toxoid.- Precipitated or adsorbed tetanus toxoid shall be prepared only from plain toxoid which meets all of the minimum requirements for plain toxoid except that it need not meet the test for potency of plain toxoid.

3.3 Test for potency of precipitated or adsorbed toxoid.- The sample used for the official potency test shall be a portion representative of the entire lot taken from the lot after the completion of all those processes of manufacture which may affect the potency of the final product. A volume of precipitated or adsorbed tetanus 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, without dilution 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 6 weeks in a 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 equal 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 test dose of the toxin shall be determined against 1/10 of a unit of the N.I.H. standard antitoxin in at least 2 guinea pigs. For a satisfactory test the control animals should die of the tetanus within an average time of not more than 4 days whereas the test animals should survive for 96 hours or more.

3.31 *Alternate method for determining the amount of tetanus antitoxin in the pooled guinea pig serums.*
The mouse test as described in Appendix A may be used for the determination of the tetanus antitoxin content of pooled guinea pig serums. Variations in the technique from that described in Appendix A may be used, provided it is shown that the results are comparable with those obtained in the guinea pig test as outlined in section 3.3.

3.4 *Volume in the final container.*- The final container of precipitated or adsorbed tetanus 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, loss in body weight, 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 32°C. with observation for not less than 7 days. For additional information refer to the Memorandum of details. (SEE AMENDMENT NO. 1)

5. *GENERAL REQUIREMENTS*

5.1 *Preservative.*- It is required that the finished product contain a preservative. However, the use of a phenoloid 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 tetanus toxoid shall be not less than 3 individual human

injections, the total volume of which shall be 3 times the volume used in section 2.3 for determining potency. The interval between injections of the plain toxoid should preferably be 3 to 4 weeks. The total human immunizing dose recommended for precipitated or adsorbed tetanus 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 potency. The interval between injections of the precipitated or adsorbed toxoid should preferably be 4 to 6 weeks.

5.3 Labeling.- The labeling of tetanus toxoid must comply with the requirements detailed in the Regulations.

5.4 Dating.- The expiration date of tetanus 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 temperature is not over 5°C.

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 of each lot by the National Institutes of Health. For this purpose the following shall be submitted:

a) A 50 ml. sample as designated in sec. 2.3 or sec. 3.3

b) A protocol similar in form to the sample shown in Appendix B or C, and giving the information indicated, including the official potency test designated in sec. 2.3 or sec. 3.3.

6. TETANUS TOXOID IN COMBINED PRODUCTS

6.1 Permissible combinations.- A combined product containing tetanus toxoid will be acceptable for release provided that it contains sufficient tetanus toxoid to comply with the total human immunizing dose requirements of sec. 5.2 and that the interval between the first and last injection is not less than the interval recommended in sec. 5.2. However, the total immunizing dose of toxoid may be divided into more individual injections than are recommended in sec. 5.2 if the other antigens present make this necessary.

6.2 Potency test of toxoid in combined products.- The official potency test of tetanus toxoid in a combined product shall be performed only after the introduction of the toxoid into the final combined product.

6.3 Volume of the 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. or more than 1.0 ml.

6.4 Volume of the final container.- The final container of a combined product containing a precipitated or adsorbed 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.- The dating of the combined product and the establishment of the date of manufacture and date of issue are determined by the antigen component having the most restrictive requirements.

6.6 Requirements for release.- Release of combined products follows the procedure prescribed in section 5.6. The protocol shall include:

a) The lot number of the product.

b) The lot number and volume of each antigen in the combined product.

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

d) The volume of each human injection.

e) A completed release protocol form for each component. This protocol form shall contain all the information required for the release of the component if it were a single antigen.

APPENDIX A

The mouse test for titration of pooled guinea pig serum for determination of its tetanus antitoxin content.

Animals: Mice 18-20 gm. weight.

Diluted Standard Toxin: The test dose of NIH standard tetanus toxin is prepared by dissolving the dried toxin in 0.85% sodium chloride solution. At present, 0.0031 gm. of standard toxin "N" per ml. is found to be satisfactory.

Diluted Standard antitoxin: A 1/50 dilution of the NIH standard antitoxin (5 units/ml.) is prepared in 0.85% sodium chloride solution.

Diluted Guinea pig serum: A 1:10 dilution of the pooled serum is prepared in 0.85% sodium chloride solution.

Test procedure: The test is run at 0.01 unit level: i.e., the control mice receive 0.01 unit of standard antitoxin plus the test dose of toxin.

Control mice: 1 ml. of diluted standard antitoxin is added to 3 ml. of 0.85% sodium chloride solution. To this mixture is added 1 ml. of diluted standard toxin.

Test mice: A series of serum-toxin mixtures is prepared in which 0.5, 0.33, 0.25, 0.20 and 0.17 ml. amounts of the diluted guinea pig serum are added to appropriate amounts of 0.85% sodium chloride solution to bring the volume of each to 4.0 ml., then 1.0 ml. of diluted standard toxin is added to complete each mixture.

General: After a combining period of 1 hour at room temperature, 0.5 ml. of each of the test and control mixtures is injected intravenously into each of three mice. All animals are observed for symptoms of tetanus until they are dead or for a period of 5 days after inoculation. The time of death is recorded in hours. For a satisfactory test the control animals should die of tetanus within 96 hours whereas the test animals should survive for 96 hours or more.

The unit value of the serum is indicated by the amount of serum contained in the toxin-serum mixture which results in survival of the test animals for at least 96 hours.

Although the supply of Tetanus Toxin (N) is limited, if used conservatively it should last for a good many years. To produce another batch of this toxin with the same characteristics may be impossible. When an ampoule is opened, the remaining toxin retains its potency held in a desiccator over H_2SO_4 under a vacuum, in the cold.

APPENDIX B

( ) Release Protocol for Products Containing Diphtheria and/or Tetanus Toxoids, Plain
( ) Diphtheria Toxoid Component ( ) Tetanus Toxoid Component

1) *Manufacturer:* _____ *Date:* _____

2) *Product:* _____ *Lot No.:* _____

3) *Manufacturing Record of Parent Toxins and Toxoids:*

Toxin Lot No.	MLD	L+	Lf	Toxoid Lot No.	%CHOH Added	%CHOH Remaining	Lf or Lb/ml.	Mg. N. Per ml.

Remarks: _____

4) *Detoxification Test (g. pigs weighing 300-400 gms.):*

Toxoid Lot No.	Date Injected	Number g. pigs	Dose	Lf/ml.	Days Observed	No. g. pigs Surv.	Non-specific deaths Number	Non-specific deaths Days Surv.

Remarks: _____

5) *Test for potency (diphtheria) (tetanus) on final product, Lot No.:* _____

Total human dose of final product is _____ ml. Used _____ gm. g. pigs.

Date of Immunization _____

Number g. pigs	Immun. Dose	Days Before Chall.	Total Chall.	No. dead	Days Survived	Total Surv. %	No. Gaining Weight	No. Losing Weight

Controls (using _____ gm ± 10% g. pigs):
2.0 ml. challenge toxin, diluted 1:10: _____ g. pigs survived ___, ___, ___, hours.

Remarks: _____

Signed: _____

Manufacturing Summary of Final Product

1) *Manufacturer:* _____ *Date:* _____

2) *Product:* _____ *Lot No.:* _____

3) *The following components were pooled so that each total human dose(_____ml.) of final product contains:*

Component and Lot Number	Amount in _____ ml.

4) *See attached protocol(s) for details of production and testing of individual components.*

5) *Date of latest satisfactory potency test for dating purposes:*

Date: _____ Component: _____

6) *The total human dose consists of _____ injections of _____ml. each.*

7) *Preservative:* _____, _____%

8) *Other tests on final product:*

Residual free formaldehyde: _____
Bulk sterility: _____
Filled container sterility: _____
Safety (guinea pig & mice): _____
Identity: _____

9) *A _____ ml. sample of the final product is being forwarded to the Laboratory of Biologics Control.*

Remarks: _____

Signed: _____

APPENDIX C

( ) Release Protocol for Products Containing Diphtheria and/or Tetanus Toxoids, Precipitated or Adsorbed

( ) Diphtheria Toxoid Component ( ) Tetanus Toxoid Component

1) *Manufacturer:* _____ *Date:* _____

2) *Product:* _____ *Lot No.:* _____

3) *Manufacturing Record of Parent Toxins and Toxoids:*

Toxin Lot No.	MLD	L+	Lf	Toxoid Lot No.	%CHOH Added	%CHOH Remaining	Lf or Lb/ml.	Mg. N. Per ml.

Remarks: _____

4) *Detoxification Test (g. pigs weighing 300-400 gm.):*

Toxoid Lot No.	Date Injected	Number g. pigs	Dose	Lf/ml.	Days Observed	No. g. pigs Surv.	Non-specific deaths Number	Non-specific deaths Days Surv.

Remarks: _____

5) *Test for potency on final product, Lot No.* _____. *Date of immunization* _____

Total human dose of final product is _____ ml. Used 450-550-gm. g. pigs.

Immunization and Bleeding						Titration of Serum Pool				
Number g. pigs	Immun. Dose	Days Before Bleeding	Number g. pigs Surv.	Number g. pigs in pool	Std* anti-toxin	Pooled serum ml.	Toxin (mg.) (ml.)	Number (mice) (g.pig)	Hrs. Surv.	Result Units in po

*N.I.H. Std. (diphtheria) (tetanus)

Control _____ Unit

Remarks: _____

Signed: _____

Manufacturing Summary of Final Product

1) *Manufacturer:* _____ *Date:* _____

2) *Product:* _____ *Lot No.:* _____

3) *The following components were pooled so that each total human dose(_____ ml.) of final product contains:*

Component and Lot Number	Amount in _____ ml.

4) *See attached protocol(s) for details of production and testing of individual components.*

5) *Date of latest satisfactory potency test for dating purposes:*

Date: _____ Component: _____

6) *The total human dose consists of _____ injections of _____ ml. each.*

7) *Adjuvant content: _____ mg. (assayed) (estimated) of _____ per individual injection.*

8) *Preservative:* _____, _____%

9) *Other tests on final product:*

Residual free formaldehyde: _____
Bulk sterility: _____
Filled container sterility: _____
Safety (guinea pig & mice): _____
Identity: _____

10) *A _____ ml. sample of the final product is being forwarded to the Laboratory of Biologics Control.*

Remarks: _____

_____

Signed: _____

APPENDIX D

Other assay methods which may be helpful to Manufacturers of Tetanus Toxoid.

1. Method for Determining the Lf units of Tetanus Toxoid

a. Reagents:

(1) Tetanus flocculating serum diluted to 50 Lf/ml.:

A reference tetanus flocculating serum is being distributed by the NIH to any laboratory which is interested in using the flocculation reaction for measuring the activity of tetanus toxin, toxoid and antitoxin. The antitoxin unit value stated on the label was determined by titration in guinea pigs against NIH standard tetanus antitoxin using the NIH tetanus toxin N. Since there is no widely used serum with which this serum can be compared by the flocculation reaction, the *in vivo/in vitro* ratio is assumed to be unity.

When setting up a working standard flocculating serum, the antitoxin unit value should be determined in guinea pigs in comparison with the NIH standard tetanus antitoxin using the NIH toxin. If other toxins are used, values may deviate by as much as 30% or 40% from that obtained when using the NIH toxin. That these variations occur is well known, but the mechanism is not as yet well understood. The flocculating value of the working standard serum should be determined by comparison with the NIH flocculating serum and the *in vivo/in vitro* ratio calculated from these two determinations.

(2) Tetanus toxoid under test

(3) M/15 phosphate buffer of pH 7.4 ± 0.05

Sodium phosphate dibasic, anhydrous ($Na_2 HPO_4$ - Reagent) 7.64 gm.

Potassium phosphate monobasic, anhydrous ($KH_2 PO_4$ - Reagent) 1.83 gm.
Distilled water. q.s. 1000 ml.

b. Procedure

Into a series of serological test tubes varying amounts of toxoid in increments of 20% are added. The total volume is then brought up to 1.5 ml. with the buffer, then 0.5 ml. of tetanus flocculating serum is added. The contents of the tubes are well mixed.

The tubes are then immersed in a waterbath at 50° C so that the height of the water in the bath comes to about half the height of the fluid inside the tubes. The tubes are placed so that the fluid can be observed with transmitted light against a dark background. Readings are made every five minutes. The *first* tube showing flocculation is the indicator tube and the time required for this flocculation is the KF. From the amount of toxoid added to this tube an estimate of the Lf content of the toxoid can be made. The test is now repeated exactly as described above with the exception that the amounts of toxoid added to the respective tubes vary in increments of 4 percent and the toxoid should be diluted so that indicator tube receives approximately 0.5 ml. of the toxoid dilution. The first tube showing flocculation indicates the amount of toxoid which is equivalent to 25 Lf units of tetanus antitoxin.

APPENDIX D

2. *Method for determining the Total Nitrogen Content of tetanus toxoid.*

The following micro-Kjeldhal procedure has been recommended by C. O Willits and C. L. Ogg of the Eastern Regional Research Laboratory, Philadelphia, Pennsylvania, and was the method followed by the Bureau of Standards in performing the total nitrogen determination on the nitrogen standard which is being distributed by National Institutes of Health.

In applying this method for estimating the purity of tetanus toxoid it is necessary to devise a means for removing the non-protein nitrogen from the sample. At the National Institutes of Health we have been dialyzing the toxoid for 24 hours in running water. Both total nitrogen content and Lf titer are determined on the dialyzed toxoid solution and the purity of the toxoid can then be expressed as Lf per milligram of protein nitrogen.

a. Reagents:

(a) Indicator

1. Methyl red-methylene blue.
Mix 2 parts of 0.2% methyl red with 1 part of 0.2% methylene blue, both in 95% ethanol.

or 2. Methyl red-brom cresol green.
Mix 5 parts of 0.2% brom cresol green with 1 part of 0.2% methyl red, both in 95% ethanol.

(b) Sodium hydroxide-sodium thiosulfate solution. ---Aqueous solution of 50 g. of NaOH and 5 g. of $Na_2S_2O_3.5H_2O$/100 ml.

(c) Boric acid solution. Dissolve 4 gm. of H_3BO_3 per 100 ml. distilled water.

c. Determination:

Measure a 1 ml. sample of dialyzed toxoid into a 30 ml. Kjeldahl digestion flask. To the sample add 1.30± 0.05 gm. potassium sulfate, 40 ± 5 mg. mercuric oxide and 2.0 ml. concentrated sulphuric acid. Add boiling chips and digest 4 hours at a vigorous boil with the acid condensing well up into the neck of the flask. Cool, add a minimum of distilled water (about 5 ml.) to dissolve the solids and place a thin film of vaseline on the lip of the flask. Transfer the digest including the boiling chips to a micro or semi-micro steam distillation apparatus of standard design. To condition the apparatus after periods of disuse, pass steam 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. Place the 125 ml. Erlenmeyer receiver containing 5 ml. of 4% boric acid and 4 drops of the indicator under the condenser with the tip extending below the surface. Add 8 ml. of the sodium hydroxide-sodium thiosulfate reagent to the still and steam distil until about 15 ml. of distillate is collected. Dilute the content of the receiver to 50 ml. with distilled water and titrate the ammonium

hydroxide with 0.02 N HCl. Choose as the end-point the grey color or the first appearance of the red. Determine the blank using the same amount of reagents rinsing the digestion flask in the same manner and using the same digestion period as that for the determination.

c. Discussion:

It will be noted that there are three main differences between this procedure and that of Sobel's method which was recommended in the National Institutes of Health memorandum of May 5, 1949. These are:

(1) Mercuric oxide is used as the catalyst.
(2) The amount of potassium sulphate is very much greater.
(3) The time of digestion is 4 hours as against 2 hours.

These changes are based upon the work of Willits and his co-workers who have studied extensively standardization of the techniques for determining nitrogen by the micro-Kjeldahl method. It is believed that two hours is too short a period of digestion and that increasing the potassium sulphate improves digestion by increasing the temperature of the digestion mixture.

The use of selenium was abandoned because it has been found that with prolonged digestion some of the nitrogenous material is converted into nitrogen instead of ammonia and results tend to be lower.

d. References:

Kjeldahl Determination of Nitrogen In Refractory Material

C. O. Willits, M. R. Coe, and C. L. Ogg
J. Assoc. of Off. Agric. Chemists 32:118 - 126: 1949 Feb.

Boiling Temperatures of Kjeldahl Digestion Mixtures

C. L. Ogg & C. O. Willits
Ibid. 33: 100-103: 1950 Feb.

APPENDIX D

3. A Method for the Assay of Free Formaldehyde in Biologic Preprations

This method is in use at the National Institutes of Health. It has given results closely comparable with those obtained by more detailed methods when used in the assay of free formaldehyde in such products as toxoids and typhus vaccine.

Details of the Method.- Dilute 1 cc. of the vaccine or toxoid with distilled water to 10 cc. Transfer 2 cc. of the diluted sample into 50 cc. Nessler tube (with about 65 cc. capacity) and dilute with distilled water to about 35 cc. Add 10 cc. diluted sulfuric acid (one vol. H_2SO_4 of sp. gr. 1.84: one vol. water) and dilute with distilled water to 50 cc. mark. Prepare similarly a series of tubes with known amounts of formaldehyde to serve as formaldehyde standards for comparison, using a 0.01 percent dilution of U. S. P. solution of formaldehyde (containing not less than 37 percent of CH_2O) and selecting quantities close to that expected in the sample. Add to the sample tube and to each of the standard tubes 10 cc. of fuchsin-sulfurous acid test solution and mix. Allow to stand one hour at room temperature and then make a colorimetric comparison of the sample tube with the standards.

Fuchsin-Sulfurous Acid Test Solution.- Dissolve 0.2 gm. of basic fuchsin in 120 cc. of hot distilled water and allow the solution to cool. Add a solution of 2 gm. of anhydrous sodium sulfite in 20 cc. of distilled water and follow by 2 cc. of hydrochloric acid. Dilute the solution with distilled water to 200 cc. and allow to stand at least 1 hour. This solution must be freshly prepared.

APPENDIX D

4. References to assay procedures found in published articles:

a - Total alum

Method for Determining Aluminum in Baking Powders: Methods of Analysis of the Association of Official Agricultural Chemists, 4th Ed., 1935, page 139.

Rapid Acidimetric Determination of Alum in Alum-precipitated Biologics, by M. B. Jacobs, J. Am. Pharm. Assoc., Sci. Ed. *39:9* 523-525, September 1950.

b. Formaldehyde

Rapid Colorimetric Determination of Formaldehyde in Toxoids by M. B. Jacobs, E. L. Eastman, and D. L. Shepard. J. Am. Pharm. Assoc., Sci. Ed. *40:8* 365-367, August 1951.

U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health
Bethesda 14, Maryland
April 8, 1954

MINIMUM REQUIREMENTS: Tetanus Toxoid, 4th Revision (December 15, 1952)

The above Minimum Requirements are hereby amended as follows, to become effective June 1, 1954:

AMENDMENT NO. 1

Section 1.2 is hereby amended to read as follows:

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.

Section 4 is hereby amended by the addition, following Section 4.3, of Section 4.4 reading as follows:

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.

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.

---

* - A control preparation of Blood Group Substances A and B may be obtained by making request to the Laboratory of Biologics Control