CLXXXIII. PURIFICATION OF DIPHTHERIA TOXOID.

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METHODS¹ for the purification of diphtheria toxoid may be divided into two classes; those which give a moderate degree of purification and a high yield, and those which give a high degree of purification and a yield which varies within wide limits. Among the latter is the method first applied to toxin by Glenny and Walpole [1915] and later modified by Watson and Wallace [1924] for toxin and by Watson and Langstaff [1926] for toxoid.

The method of Watson and Langstaff depends on the fact that toxoid is precipitated from aqueous solution in the p_H range 3.8 to 4.4. The precipitated toxoid is in a high state of purity relative to crude toxoid solution but yields may vary with different toxoid preparations from 5 to 90 %.

It seemed reasonable to assume that the low yields which are often obtained by this method might be due not to true solubility of toxoid in the acid solution but either to a slow rate of precipitation or to the presence of substances in the solution which interfere with precipitation. Granting the correctness of either of these suppositions, a toxoid once precipitated should not redissolve in a solution of p_H 3.8 to 4.4. The work which was involved in attempts to prove or disprove this assumption regarding the solubility of toxoid within a certain range of acidity resulted in the methods of purification which are described in this paper. In brief, these methods depend upon precipitation of toxoid in the p_H range 3.8 to 4.4 by precipitating agents which may also precipitate much other material, followed by removal, under the same conditions of acidity, of the precipitating agent and consequently of other material soluble within the range of insolubility of the toxoid.

METHOD I.

Precipitation by ferric chloride.

If to a toxoid sufficient ferric chloride solution be added and the reaction of the mixture adjusted to p_H 4.2 a heavy brownish coloured precipitate which contains most of the toxoid is formed. If now a citrate buffer p_H 4.2 in the proper amount be added to the whole mixture the greater part of the precipitate will shortly disappear leaving a small amount of lighter coloured precipitate. The purity of the toxoid contained in this precipitate is of the order of that obtained by the method of Watson and Langstaff but the yields are consistently higher. Various steps in the method will be dealt with under separate headings.

(a) *The amount of ferric chloride required for maximum precipitation.* The results shown in Table I were obtained as follows. 3.5 % ferric chloride solution was added in varying amounts to 50 ml. portions of toxoid solution containing 1 % sodium acetate; the reaction of each mixture was adjusted to p_H 4.2 by the addition of *N* HCl. After about 1 hour the solutions were centrifuged, the supernatant liquids poured off and the precipitates dissolved in water

¹ A fairly complete review of existing methods has been given in a recent paper by Leonard and Holm [1933].

Table I. *Precipitation of 50 ml. portions of toxoid by ferric chloride.*

Ferric chloride ml. 3.5 %	Yield %
0.4	70
0.8	74
1.6	84
3.2	100
6.4	91

by the addition of solid sodium citrate. The amount of sodium citrate added was such as to give a final concentration of 2 %. A short heating at 45° was used to facilitate solution. The toxoid content of the solutions was then determined by the flocculation method. M. L. Smith [1932] had shown that sodium citrate could be used to dissolve the precipitate formed by the addition of alum to toxoid and that such a solution gave a specific flocculation with antitoxin. Citrate can also be used to dissolve the precipitate formed by the addition of ferric chloride to toxoid, and the solution so formed will flocculate specifically with antitoxin. The data given in the table show that for 50 ml. of the toxoid preparation used, 3.2 ml. of the ferric chloride solution were sufficient for precipitation of the toxoid. The minimum amount of ferric chloride required for the precipitation of toxoid varies with different preparations but for the toxoids which we have used it has been found that ferric chloride in the proportion of 1 vol. of 3.5 % solution to 10 vols. of toxoid solution precipitates the maximum amount of toxoid.

(b) *The adjustment of the p_H .* For convenience in adjusting the p_H of the toxoid solutions solid sodium acetate was added to a concentration of 1 %. This was done before the addition of ferric chloride and hydrochloric acid. The reactions of the solutions were determined by the spot plate method of Haas [1919] using bromocresol green as indicator. Determinations by this method were checked from time to time by measurements with a glass electrode¹.

(c) *Citrate buffer. The amount and the manner of addition.* In order to remove iron from the ferric chloride precipitate, a concentrated citrate buffer was used. This was prepared by mixing in the proper proportions the following solutions: (1) sodium citrate 307 g. per litre and (2) citric acid 420 g. per litre. The p_H of the concentrated buffer prepared from these two solutions was determined after diluting 20 times with water. With the chemicals used by us a mixture in the proportion of 1 ml. of the sodium citrate solution to 0.65 ml. of the citric acid solution gave when diluted 20 times with water approximately p_H 4.2.

In addition to p_H there are two other factors which may affect the yield of toxoid obtained by extraction of the ferric chloride precipitate with citrate buffer, *viz.* the amount of buffer and the rate at which the buffer is added. In Table II are summarised the results of an experiment on the effect of the addition of different amounts of citrate buffer to toxoid precipitated with ferric chloride. Five samples of a toxoid, 50 ml. each, were precipitated with ferric chloride and adjusted to p_H 4.0. After these solutions had stood for 1 hour different amounts of citrate buffer p_H 4.0 were added quickly and the mixtures kept another hour. The undissolved precipitates were removed by centrifuging, dissolved in water and the Lf values determined. The results in Table II show that the addition of 2 ml. of citrate buffer gave a high yield of purified toxoid and that the addition of larger amounts of citrate buffer resulted in lower yields.

¹ In measuring the E.M.F. of the glass electrode-calomel chain, a circuit arrangement of the type described by V. G. Smith [1934] was used.

Table II. *Effect of citrate buffer on yields.*

Conc. citrate buffer p_H 4·2 ml.	Yield %	Nitrogen mg./Lf
0	95	0·0350
1	95	0·0048
2	95	0·0020
4	79	0·0021
8	75	0·0021

The lower yields resulting from the addition of larger amounts of citrate buffer were found to depend on the rate of addition of the citrate. This is shown by the following experiment. Two 50 ml. samples of toxoid were precipitated with ferric chloride and the p_H of each was adjusted to 4·2. After 1 hour 16 ml. of citrate buffer, p_H 4·2, were added quickly to one sample and to the other the buffer was added fractionally 1 ml. at a time with an interval of 10 minutes between additions. An hour after the last addition of buffer, the two solutions were centrifuged, the precipitates removed, dissolved in water and the Lf values determined. The results are shown in Table III.

Table III. *Effect on yield of rate of addition of citrate buffer.*

Conc. citrate buffer p_H 4·2 ml.	Manner of addition of buffer	Yield %
16	Quickly	68
16	Slowly	85

The following are the details of the ferric chloride method of purification which we have applied to a number of toxoid preparations.

1 g. of sodium acetate is dissolved in 100 ml. crude toxoid and to this 10 ml. of 3·5 % ferric chloride solution are added; *N* HCl is then added to p_H 4·2 and the mixture is kept for 1–2 hours. Next, 4 ml. concentrated citrate buffer, p_H 4·2, are added drop by drop with constant stirring over 30 minutes and the solution is again kept for 1–2 hours. The undissolved precipitate is removed by centrifuging, washed with dilute (1 in 20) citrate buffer p_H 4·2 and dissolved in saline by the careful addition of dilute NaOH to p_H 7·0–7·5. Using the various chemicals in the proportions just set forth, we have successfully applied this technique to relatively large lots of toxoid, up to 17 litres per lot.

It is important to note that the ferric chloride and the citrate should be added to the toxoid in the order given. If, for example, citrate is added before ferric chloride a low yield of toxoid may result.

When purifying large amounts of toxoid we have used a Sharples centrifuge, the bowl of which was lined with cellophane to facilitate the collection of the precipitate.

Table IV. *Yields by ferric chloride-citrate method.*

Lot No.	Yield %	Lot No.	Yield %	Lot No.	Yield %	Lot No.	Yield %
531	90	649	100	220	98	680	100
705	87	0529	94	524	84	651	100
723	81	0487	80	525	85	515	100
573	90	556	85	A	82	722	94
590	72	272	75	257	95	748	100
660	85	279	83	609	87	230	86
687	77	643	86	694	100	589	100

Yields obtained by this ferric chloride-citrate method are shown in Table IV and Table V gives a comparison of the yields obtained by acid precipitation and by the ferric chloride-citrate method.

Table V. *Comparison of yields by acid precipitation and ferric chloride-citrate method.*

Toxoid	Acid-pptn.	FeCl ₃ -citrate	Toxoid	Acid-pptn.	FeCl ₃ -citrate
	Yield %	Yield %		Yield %	Yield %
257	36	95	515	40	100
609	57	87	525	30	85
694	35	100	722	41	94
680	24	100	748	32	100
651	32	100			

Table VI gives data on the composition of purified toxoid obtained from two different toxoid preparations by the ferric chloride-citrate method.

Table VI. *Analytical data for two purified toxoids.*

Yield %	Lf/ml.	Total solids mg./ml.	Ash mg./ml.	Fe mg./ml.	Nitrogen mg./Lf
77	100	2.16	0.065	0.033	0.0022
76	100	0.84	0.22	0.005	0.0007

Other workers have reported values for the nitrogen content of acid-precipitated toxoid ranging from 0.0006 to 0.009 mg./Lf. The data in the table show that toxoid purified by the method described above is as pure as that obtained by the acid precipitation method.

With a few lots of toxoid we have compared the yields obtained with the ferric chloride-citrate method and with the same method in which ferric chloride was replaced by potassium alum. The yields with potassium alum were consistently lower than with ferric chloride.

METHOD II.

Precipitation by acetone.

The principles involved in this method of purification are the same as those in the ferric chloride-citrate method.

The following are details. Sodium acetate is added to crude toxoid to give 1 % concentration and the reaction of the solution is adjusted to p_H 4.2 with *N* HCl. The toxoid is then precipitated by the addition of 2 vols. of acetone, the precipitate being separated by centrifuging and the supernatant fluid poured off. Part of the supernatant fluid (1/30 vol.) is returned to the precipitate and to the resulting mixture acetate buffer, p_H 4.2, in amount equal to 1/5 the volume of the original toxoid solution is added slowly and with constant stirring. The mixture is kept for 1 hour and acetate buffer is again added in amount sufficient to make the total volume of the acetate buffer equal to one-half the volume of the crude toxoid solution. The small amount of precipitate which remains undissolved is purified toxoid. This precipitate is separated by centrifuging, washed with acetate buffer, p_H 4.2, and dissolved in water with the addition of sodium hydroxide.

If good yields are to be obtained it is important that those substances contained in the acetone precipitate which are soluble in water at p_H 4.2 dissolve

slowly in the acetate buffer. This is accomplished by the technique which has already been described *viz.* by returning some of the acetone supernatant fluid to the precipitate and by slowly adding acetate buffer. Once the water-soluble substances have been removed, the purified toxoid is insoluble in acetate buffer, p_H 4.2.

The importance of the manner of addition of acetate buffer to the acetone precipitate is shown in Table VII.

Table VII. *Extraction of acetone precipitate by two additions of acetate buffer.*

Acetate buffer p_H 4.2		Yield %
1st addition ml.	2nd addition ml.	
1	49	36
2	48	39
4	46	71
8	42	75
16	34	71
32	18	57
Acetone precipitate not extracted		75

The data for this table were obtained as follows: 50 ml. portions of toxoid were precipitated by acetone in the manner already described. The mixtures were centrifuged and the supernatant fluids poured off and to each precipitate were added 5 ml. of the acetone supernatant fluid and the amount of acetate buffer, p_H 4.2, shown in the table under "1st addition." The precipitates were broken up, stirred with the liquid and kept for about 1 hour. Acetate buffer was again added to each mixture to give a final volume of 50 ml. Finally the precipitates were removed by centrifuging and dissolved in saline by the addition of sodium hydroxide.

It should be noted that the final concentrations of acetone and acetate were the same for all the solutions but that the best yields were obtained in those cases in which 4–16 ml. of acetate buffer were first added to the 5 ml. of acetone supernatant fluid.

Table VIII gives results of the acetone method of purification with a number of toxoid preparations.

Table VIII. *Yields by acetone-acetate buffer method.*

Toxoid No.	Yield %	Toxoid No.	Yield %	Toxoid No.	Yield %
272	80	750	74	769	82
680	80	220	73	643	86
717	83	730	94	279	74
939	96	768	82	H.S.	86
749	87				

Diphtheria toxoid may also be purified by a technique similar to that given above, in which ethyl alcohol (5 vols.) is used in place of acetone (2 vols.).

METHOD III.

Adsorption by ferric phosphate.

A number of methods have been described for the purification of diphtheria toxin and diphtheria toxoid which depend on adsorption on certain insoluble substances and elution of the adsorbed toxin or toxoid by a suitable solvent [Abt, 1928; Gross, 1929; Ohyama, 1931; Schmidt *et al.*, 1931; Siebenmann, 1932].

Since ferric phosphate is insoluble at p_H 4.2 and since it may be dissolved in citrate buffer we decided to investigate the possibility of the application of the principles already described to the purification of toxoid by adsorption on ferric phosphate.

A suspension of ferric phosphate was prepared by mixing 35 ml. of 3.5 % ferric chloride and 10 ml. of 10 % potassium dihydrogen phosphate and adjusting to p_H 4.5 by the addition of sodium hydroxide. The resulting precipitate was removed by centrifuging, washed twice with acid water, p_H 4.5, and suspended in sufficient saline to give a volume of 70 ml. and the p_H adjusted to 4.2.

10 ml. amounts of this suspension were added to 50 ml. amounts of four different toxoid preparations to each of which had been added 0.5 g. of sodium acetate. Hydrochloric acid was then added to each mixture to p_H 4.2 and these were kept for 3 hours with frequent shakings. The suspensions were centrifuged and the precipitates washed once with saline, p_H 4.2, and resuspended in 40 ml. of saline. To each of these suspensions 8 ml. citrate buffer, p_H 4.2, were added. The buffer was added slowly 1 ml. at a time with an interval of about 10 minutes between each addition. By this treatment the greater parts of the precipitates were dissolved. The undissolved portions were removed by centrifuging and dissolved in saline by the addition of NaOH to p_H 7.0-7.5.

The yields of toxoid obtained are shown in Table IX along with the yields on the same toxoids purified by the ferric chloride-citrate method.

Table IX.

Toxoid No.	Ferric chloride-citrate	Ferric phosphate adsorption
	Yield %	Yield %
220	100	100
52	75	70
680	85	80
643	80	85

It is of some interest to note that for the particular adsorption complex, ferric phosphate-toxoid, we were able to separate the adsorbed toxoid by dissolving the adsorbing substance leaving the toxoid undissolved rather than by separating the toxoid with a suitable eluent.

Table X gives a comparison of the purifications effected by the different procedures which have been described.

Table X.

Treatment	Yield %	Nitrogen mg./Lf
Original toxoid	—	0.160
FeCl ₃ —precipitate	100	0.0518
FeCl ₃ ppt.—extracted	93	0.00125
Alcohol—precipitate	93	0.0279
Alcohol ppt.—extracted	73	0.00088
Acetone—precipitate	93	0.0367
Acetone ppt.—extracted	83	0.00111
Ferric phosphate	88	0.00077

It will be seen that the degree of purification is of the same order for all three methods.

SUMMARY.

Methods of purification have been described by which consistently good yields of diphtheria toxoid in a high state of purity may be rapidly separated from solutions of crude toxoid. These methods consist essentially of precipitation of toxoid by certain precipitating agents and removal from the precipitate of material soluble in the acid range of insolubility of the toxoid.

Toxoids purified by the methods described have been used on laboratory animals both as primary and secondary stimuli. The antigenic response, Lf per Lf, was of the order of that obtained with unpurified toxoids.

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