63. Purification of Tetanus Toxoid

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There have been a number of reports [Parish & Oakley, 1940; Whittingham, 1940; Cooke, Hampton, Sherman & Stull, 1940], some unpublished, of anaphylactoid reactions in man following a second injection of tetanus toxoid. These reactions have been ascribed to proteoses or peptones present in the toxoid preparations; and it is highly probable that such constituents were responsible for the reactions observed. There is, however, the possibility that tetanus toxoid itself could serve as a sensitizing and shock-inducing agent and in view of this it was decided to explore the possibility of producing a suitable insoluble suspension of toxoid by precipitation, for example with alum or protamine [Moloney & Orr, 1938], in order to decrease its shock-producing properties. To make such an insoluble product it is desirable to have the toxoid in a relatively high state of purity, and to this end the method of purification which is described here was developed.

The method consists of first concentrating the toxoid solution and then precipitating the active material from the concentrated solution in the pH range 4.0–4.4. It might be objected, on the analogy of results obtained with diphtheria toxoid [Linggood, 1941], that subjecting tetanus toxoid to this degree of acidity would damage the toxoid protein. There is, however, no evidence of such damage in the experimental results which are presented.

EXPERIMENTAL

Details of the method

To 1500 ml. of crude toxoid contained in a 3-litre Erlenmeyer flask, 950 g. of ammonium sulphate are added. The flask is shaken until the ammonium sulphate is dissolved and then left overnight at room temperature. The solid which separates and floats to the surface of the liquid contains the greater part of the toxoid. The liquid is removed by careful decantation, and as it is poured off, the solid tends to adhere to the sides of the flask. We have never had occasion at this stage to resort to filtration. When the separation is completed, the precipitate remaining in the flask is dissolved in about 80 ml. of distilled water.

From this concentrated solution (50 ml. amount) the toxoid is precipitated by the addition of solid ammonium sulphate (30 g./50 ml. dialysed solution or corresponding amount calculated from Table 1). Acetate buffer pH 4.2 (125 ml.) is then stirred in over about 1 min. The mixture is at once centrifuged and the precipitate which contains the greater part of the toxoid is dissolved in about 30 ml. distilled water plus about 0.5 ml. 5% NaHCO_3 (to pH 7.0) and made up to 50 ml. The whole operation from the addition of the acetate buffer until the solution in bicarbonate should be completed within 10 min. The dissolved toxoid is centrifuged to free it from insoluble material and then dialysed against running water.

With the order of addition of ammonium sulphate and acetate buffer given above the best yields were obtained. There are however certain toxoid preparations, for example those containing Witte peptone, which on precipitation with ammonium sulphate yield sticky precipitates; these are not conveniently extracted with acetate buffer, and for such preparations it is preferable first to dissolve the ammonium sulphate in the buffer and then to add this solution to the concentrated toxoid.

In the above procedure it is required that ammonium sulphate be added to the concentrated solution of toxoid in the proportion of 30 g./50 ml. of ammonium sulphate-free toxoid solution. To this end, either the concentrated solution of toxoid can first be freed from ammonium sulphate by dialysis or the amount of ammonium sulphate required to be added can be determined from the specific gravity of the solution and the data given in Table 1. The figures given in the table are not absolute values, since, for example, there is a variation in the specific gravity of salt-free toxoid, but they are sufficiently accurate for the purpose in hand.

Table 1. *Specific gravity and ammonium sulphate required*

Specific gravity	g. (NH ₄) ₂ SO ₄ required per 50 ml. toxoid	Specific gravity	g. (NH ₄) ₂ SO ₄ required per 50 ml. toxoid
1.020	30.0	1.065	24.5
1.035	28.0	1.080	23.0
1.050	26.5		

N values of purified toxoids

The purities recorded in Table 2 may be compared with the Lf-nitrogen values for the crude toxoids which were purified, namely values ranging from 3.3 Lf/mg. N to 5.0 Lf/mg. N, and with the value for toxoid of 3300 Lf/mg. N derived from an analysis of

Table 2. *Yields and purities*

Toxoid	Original Lf/ml.	Purified Lf/ml.	Yield %	Purified Lf/mg. N
155	17	160	70	835
160	8	165	70	530
170	13	135	86	435
171	15	117	65	770
172	6	96	80	270
173	5	80	73	250
174	15	190	70	770
644	7	80	75	670
67	7	53	75	69

tetanus toxoid-antitoxin floccules [Moloney & Hennessy, 1942]. The method used in obtaining this latter figure was that described by Pappenheimer & Robinson [1937] for the analysis of diphtheria toxin-antitoxin floccules.

Table 3. *Reactivity of purified toxoids*

Toxoid	Peptone in crude toxoid	Result of purified toxoid intravenously	Toxoid	Peptone in crude toxoid	Result of purified toxoid intravenously
155	Martin's peptone	No anaphylaxis	173	Martin's peptone	No anaphylaxis
160	"	"	174	"	"
170	"	"	644	Neopeptone	"
171	"	"	67	Witte peptone	Mild anaphylaxis
172	"	"			

Preparation no. 67 gave decidedly the poorest degree of purification. This result is in accord with the finding for the same toxoid, recorded in Table 3, namely that Witte peptone had not been effectively removed.

Presence of peptones in purified toxoids

Since it was highly probable, as noted above, that in some instances certain peptones were responsible for shock following a second injection of tetanus toxoid, it was of interest to test for the presence of these substances in the solutions of purified toxoid. This was

done by injecting the purified toxoids into guinea-pigs sensitized with the suitable peptones and noting whether or not anaphylaxis occurred.

The guinea-pigs which were used were sensitized as described by Moloney & Orr [1938] in testing diphtheria prophylactics. In the cases of Witte peptone and Neopeptone a 2% solution of the peptone was made and to this was added potassium alum to give a concentration of 1%. The pH of the solution was then adjusted with 5% Na_2CO_3 to approximately 6.0 and 5.0 ml. amounts of the suspension were injected subcutaneously into each of a number of guinea-pigs ranging in weight from 350 to 450 g. Six weeks after the injection of the alum-precipitated peptone the animals were ready for use. For sensitizing animals with Martin's peptone the same procedure was employed except that Martin's broth was used instead of a 2% peptone solution.

In carrying out a test the purified toxoid was first diluted to the same Lf value as the original toxoid and 1.0 ml. was injected into a femoral vein after exposure of the vein under local anaesthesia. The animal was observed for about 10 min. and if no reaction occurred its sensitivity was tested for by injecting 1.0 ml. of the original toxoid into the other femoral vein. In Table 3 'no anaphylaxis' means that following the intravenous injection of the purified toxoid there was no symptom of anaphylaxis, but that in the same animal following the intravenous injection of the original toxoid there was typical fatal anaphylaxis.

It should be noted that the presence of peptone in purified toxoids does not necessarily render these unsuitable for conversion into insoluble antigens, since the shock-producing properties of both peptone and toxoid are greatly lessened by precipitation with alum or other suitable compound.

Time of flocculation of purified toxoids

There are set forth in Table 4 the Kf values of original toxoids and of the corresponding purified toxoids. These latter were diluted to the original Lf values before flocculation.

Table 4. *Kf values of original and of purified toxoids*

Toxoid	Lf/ml. original and purified	Kf 46° original min.	Kf 46° purified min.
155	17	100	75
160	8	270	115
170	13	165	85
171	15	160	75
172	6	255	145
173	5	240	210
174	15	130	75
644	7	150	90
67	7	700	360

In every instance the times of flocculation of the purified toxoids are less than those of the original toxoids. On the basis of experience with diphtheria toxoid this result was unexpected since during purification the toxoids were exposed to an acidity represented by pH 4.2. Hence one might have looked for an alteration in the toxoid protein which would have been made manifest by an increase in the time of flocculation. One cannot of course conclude from the results which were obtained (Table 4) that the toxoids had suffered no damage; it is possible that the decrease in time of flocculation of the purified toxoids is due to the removal, during purification, of substances which tend to inhibit flocculation, a possibility which we have not explored.

Tetanus toxoid purified by the method described here has been used on a small scale to immunize animals and man, but as yet we have no adequate comparisons of the relative antigenic efficacies of purified and unpurified toxoids.

It is well known that flocculation with mixtures of tetanus toxin (or toxoid) and anti-toxic sera may occur in more than one zone and that with, for example, a single serum

and a single toxoid, it is not possible by flocculation alone to decide which is the true toxoid-antitoxin zone. This difficulty can present itself with purified toxoid but in practice it has not arisen during a year in the routine testing of trial bleedings from horses undergoing immunization.

SUMMARY

A simple method is described for the preparation of purified and concentrated solutions of tetanus toxoid. Purified toxoids made from broths containing Martin's peptone or neopeptone ranged in purity from 250 to 830 Lf/mg. N as compared with 3.3 Lf/mg. N to 5.0 Lf/mg. N for the original toxoids and 3300 Lf/mg. N as a probable value for pure toxoid.

The method of purification did not rid the toxoid of Witte peptone when this constituent was present in the crude toxoid. On the other hand crude toxoids containing Martin's peptone and neopeptone yielded products in which these peptones could not be detected.

The purified toxoids flocculated in less time than did the corresponding crude toxoids.

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