THE PURIFICATION AND CONCENTRATION OF DIPHTHERIA TOXOID

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Owing to the danger of sensitization by toxin-antitoxin mixture, the use of toxoid in the immunization of children has become quite general. The importance of making available a procedure for the preparation of a diphtheria toxoid which, without giving rise to more than negligibly slight reactions, will induce an immunity equivalent to, or greater than, that conferred by the mixture, suggested the application of those methods of purification that were so successful when used for the toxins of the hemolytic streptococci (1) to the preparation of diphtheria toxoid.

A refined toxoid was first obtained by Watson and Langstaff (2) by the acetic acid precipitation method which Watson and Wallace had applied to the concentration of toxin. Kept in the cold room, it remained stable for more than 2 years. Fitzgerald (3) mentions that toxoids purified by this method, and by a modification of it, were observed to be very much less stable than the unconcentrated material.

Later, S. Schmidt (4), by adsorption on aluminium hydrate in suspension, obtained a concentrated toxoid with 600 flocculating units per cubic centimeter, the reactions to which were less severe than those induced by the routine material. The procedure is difficult and time-consuming, and the loss so great that the cost is prohibitive for production on a large scale, unless, as the author suggests, sufficient immunity can be induced by one dose. Schmidt (5) also used hydrochloric acid but found the purified toxoid less antigenic than that purified by adsorption on aluminium hydrate. Moreover, the acid-precipitated material gave rise to local reactions in the skin of guinea pigs.

By precipitation with hydrochloric acid, Bunney (6) recently prepared a purified diphtheria toxoid which did not give rise to any local reactions in 50 adults.

Our preliminary experiments revealed that, with acetone precipitation at 4°C., the formalin was immediately and completely eliminated

without injury to the toxoid; a fact that at once provides a basis for the development of improved methods of detoxification, standardization, and accurate control in the preparation of diphtheria toxoid.

The toxin broth used in the first experiment was obtained from the growth of the diphtheria bacillus in medium prepared with 2 per cent of peptone, phosphate salts, and ammonium lactate, but without meat infusion (7). The M.L.D. was -0.003 cc. and the L_+ dose was 0.16 cc. The toxoid was prepared by the addition of approximately 0.3 per cent of formalin and incubation at 39°C. until the material was completely detoxified. The 14 liters were in four containers numbered 38-I, 38-II, 38-III, and 38-IV, which were kept separate throughout the experiment. The toxoid was then concentrated by precipitation with acetone:

> The toxoid and U.S.P. acetone were cooled separately in an ice bath to about 4°C. Twice the volume of acetone was then added to the toxoid which was shaken meanwhile. The acetone, after about 15 minutes, was separated from the precipitated toxoid by decantation. The flasks were then inverted and the acetone allowed to drain. When the odor of acetone was no longer detectable, a small amount of distilled water was added to the flasks and the precipitate dissolved. The solution was then placed in an ice bath for about an hour. At this temperature the insoluble phosphates readily settled. The solution was then centrifugalized and the supernatant liquid dialyzed for 72 hours at from 4 to 8°C. The process of precipitation and resolution was repeated. The toxoid solution was placed in a small dish and dried *in vacuo* over sulfuric acid.

The dried product was dissolved in sufficient 0.85 per cent physiological salt solution to restore it to one-half of its original volume. The flocculating value was approximately fifteen antigenic units per cubic centimeter. The acetone had removed all of the formaldehyde and a preservative was necessary. Enough phenol was added to lots numbered 38-I and 38-II to make the concentration 0.4 per cent. To lots numbered 38-III and 38-IV, sufficient merthiolate was added to make the dilution 1:10,000. The four lots of toxoid were then filtered through Mandler filters. The pH values, nitrogen determinations, flocculating units, and time of flocculation are recorded in Table I.

The nitrogen in the purified toxoid, as determined by the Kjeldahl method, showed a reduction of about 62 per cent. The toxoids con-

taining the merthiolate as a preservative were slightly more alkaline than were those containing phenol, but the pH of all was satisfactory; none required adjustment. According to the flocculating values obtained with the concentrated material, about 15 per cent of the total antigenic content was lost during the operation. The time of flocculation, however, was reduced about one-half. It is generally recognized that this reaction is influenced by several factors: the colloidal properties of the toxoid, the presence of free formaldehyde, the concentration of antigenic units, and the hydrogen ion concentration.

TABLE I

Comparison of the Unconcentrated and Refined Diphtheria Toxoids

Toxoid	Total volume	pH	Kjeldahl nitrogen	Flocculating value*			Preservative
					Time		
	cc.		*mg. per 100 cc.*	*units per cc.*	*hr.*	*min.*	
Unconcentrated, No. 38 (I, II, III, IV)	13,850	6.9	335.7	9.0	1	45	
Refined, No. 38-I	1,800	7.1	129.9	15.6		51	Phenol 0.4 per cent
Refined, No. 38-II	1,300	7.1	130.1	16.8		50	Phenol 0.4 per cent
Refined, No. 38-III	1,900	7.5	124.4	14.8		55	Merthiolate 1:10,000
Refined, No. 38-IV	1,900	7.4	130.9	15.6		58	Merthiolate 1:10,000

* Flocculation tests were done in water bath maintained at 40°C.

It is difficult to suggest to what extent, or how, any or all of these conditions affect the reactions obtained with this refined product but the formalin was eliminated in the process and the total volume was half that of the original material. In so far as could be determined from these tests, the toxoid obtained by this comparatively simple method, with relatively slight loss, was highly satisfactory. Certain important properties, however, remained to be studied in connection with its value as an immunizing agent.

In earlier attempts to purify diphtheria toxin by this method we found that after 20 hours' exposure, and the resultant precipitation, the acetone destroyed about 70 per cent of the toxic fraction of diphtheria toxin, while the paralyzing properties, Ehrlich's "toxones," were retained. This result, from the standpoint of detoxification of diph-

theria toxoid, was important and was confirmed later by our experience in the purification of toxoid. A toxoid, No. 41, which, unconcentrated, had caused slight paralysis in guinea pigs injected with 5 cc. amounts, continued to do so when concentrated and restored to its original volume. Thus, although the action of the acetone on the toxin might suffice to complete the detoxification resulting from the action of the formalin, it could not be relied upon to prevent the paralysis induced by a partially detoxified toxoid. Further studies will be required to compare the detoxification accomplished by the two reagents, formalin and acetone, but for present purposes the results of these experiments indicate the importance of complete detoxification by the treatment with formalin before concentration by precipitation with acetone. It is also important to determine the optimum percentage of formalin and optimum period of exposure for this detoxification, since the immediate and complete removal of the formalin by the acetone precipitation opens up the possibility of greatly improving this important step in the preparation of the toxoid. These studies are in progress. When the detoxification is complete the refined product obtained by acetone precipitation, when injected in 5 cc. amounts into the guinea pig, does not give rise to paralysis.

One very serious criticism of the refined product obtained by other methods of purification has been the lack of stability, as reported by Fitzgerald (3), and H. Schmidt (8). This criticism has applied not only to methods in which acid, but also to those in which acetone, precipitation was used. S. Schmidt (9), in a comparison of the stability of purified toxoid with purified toxin, by the flocculation reaction, found the toxoid more stable than the toxin purified by the same processes—aluminium hydrate adsorption and hydrochloric acid precipitation. However, data as to the stability of the purified toxoid over a period of months were not given. Our observations are limited to a period of 7 months during which toxoid No. 38-II containing 0.4 per cent phenol was tested at intervals after storage in the cold room. No change was detected in its flocculating value, the time of flocculation, nor in the immunity index test in guinea pigs.

The effect of exposure at different temperatures is also significant. The results of these experiments are recorded in Table II in which the original toxoid and the refined product, with and without preserva-

TABLE II

Comparison of the Stability of the Unconcentrated and Refined Toxoids with and without Preservative

Toxoid	Preservative	Temperature*	Original flocculating value			Final flocculating value		
				Time			Time	
		°C.	*units per cc.*	*hr.*	*min.*	*units per cc.*	*hrs.*	*min.*
Unconcentrated, No. 9-B..		−10	19.2		25	19.2		25
Unconcentrated, No. 9-B..		39	19.2		25	19.2		25
Refined, No. 162.....		−10	29.1		25	29.1		25
Refined, No. 162.....	Merthiolate 1:10,000	−10	29.1		25	29.1		25
Refined, No. 162.....	Phenol 0.4 per cent	−10	29.1		25	5.1	23	45
Refined, No. 162.....	Phenol 0.4 per cent	39	29.1		25	29.1		25

* All toxoids maintained at stated temperatures for 20 hours.

tive, were exposed for 20 hours at 39°C. and at −10°C., without alteration in the flocculating value of any of the toxoids, with the one exception of the purified toxoid preserved with 0.4 per cent phenol. The flocculating value of the phenolized toxoid was almost completely destroyed at −10°C. after 20 hours' exposure. 5 cc. of the material which had been frozen were injected subcutaneously into a guinea pig and induced no symptoms of toxic poisoning. The effect of exposure at low temperatures thus depends apparently upon the preservative used; further investigation is needed to determine the changes in toxicity, flocculating values, or antigenic action in the tissues, of preparations which are to be used for preventive inoculation. Dissociation of toxin-antitoxin mixtures after freezing has been noted by White and Robinson (10), whereas Kirkbride and Dow (11) in their experiments with 1/10, 1, 3, and 5 L_+ mixtures of toxin and antitoxin exposed at temperatures from −5.6°C. to −17°C. for periods of from 3 to 72 hours failed to detect increased toxicity as evidence of dissociation. On the contrary, the results indicated a decrease in toxicity, possibly due to the destructive action of phenol upon the toxin after dissociation. Reduction in the immunizing properties, and dissociation of frozen toxin-antitoxin mixtures preserved with phenol, have been stressed by several observers—Harrison (12), Anderson and Leonard (13), and Glenny, Pope, Waddington,

and Wallace (14); while Watson and Langstaff (2) observed a lowering of the flocculating value of frozen purified toxoid preserved with phenol. The concentration of phenol and its affinity for the protein present in the mixtures, appear to be fundamental factors which determine the results obtained in these different experiments but it is not yet altogether clear just what reactions take place and how they affect the toxicity of the mixtures on the one hand, or the flocculating and antigenic values of the toxoid on the other.

Although flocculation and the time within which it occurs have been considered indications of the antigenic activity of a toxoid as an immunizing agent in the tissues, a series of experimental studies was made to determine, by tests in animals, the antigenic action of a refined toxoid in the tissues as compared with that of a toxin-antitoxin mixture and a formol toxoid, and to ascertain whether, and how closely, the results of the flocculation test would correspond to those of animal inoculation.

The Comparative Antigenic Action of Toxin-Antitoxin Mixture, Toxoid, and Refined Toxoid as Immunizing Agents

This comparative study was undertaken with toxoids which were prepared from the routine veal infusion broth and from the infusion-free medium, No. 25 and No. 5, respectively, with flocculating values of 7.0 and 7.2. The refined toxoid No. 5-R was prepared from No. 5; the volume was half that of the original toxoid. It had a flocculating value of 11.7. The toxin-antitoxin mixture selected, No. 670, was so prepared and standardized that 1 cc. contained 1/10 of an L_+ dose of veal infusion diphtheria toxin.

One set of guinea pigs received two doses of 0.5 cc. of each antigen with an interval of a week between injections. A second set of guinea pigs was injected with one dose of 1 cc. of each antigen. 1 month after the last injection the animals were bled from the heart and the antitoxin in the blood was titrated intracutaneously with a standard toxin (15). A month following the first bleeding, the guinea pigs were bled again and the antitoxin was titrated as before. 10 weeks after the last injection, the immunity of all these guinea pigs was titrated by the intracutaneous injection of varying dilutions of standard toxin—0.04, 0.1, 0.2, 0.5 of an M.L.D. The results are recorded in Tables III and IV.

TABLE III

Titration of Antitoxin in the Blood of Guinea Pigs Immunized with Diphtheria Toxoid or Toxin-Antitoxin Mixture

Antigen	Method of immunization (subcutaneously)	Animal No.	Units of antitoxin		Average of antitoxic units	
			After 4 wks.	After 8 wks.	After 4 wks.	After 8 wks.
Refined toxoid No. 5-R	1 cc. in 1 injection	1736	+1/100 −1/20	+1/100 −1/20	+1/100	+1/100 −1/20
		1737	1/100	+1/100 −1/20		
	2 doses of 0.5 cc.	1711	1/100	1/20	1/100	1/20
		1710	1/100	1/20		
Veal infusion toxoid No. 25	1 cc. in 1 injection	1740	+1/500 −1/100	+1/100 −1/20	1/100	+1/100
		1742	1/100	1/100		
	2 doses of 0.5 cc.	1723	+1/500 −1/100	+1/100 −1/20	+1/500 −1/100	+1/100
		1724	+1/500 −1/100	1/100		
Infusion-free toxoid No. 5	1 cc. in 1 injection	1745	+1/100 −1/20	+1/100 −1/20	1/100	+1/100
		1746	+1/500 −1/100	−1/100		
	2 doses of 0.5 cc.	1728	+1/500 −1/100	+1/100 −1/20	1/100	+1/100
		1726	1/100	+1/100 −1/20		
Toxin antitoxin-mixture No. 670	1 cc. in 1 injection	1753	−1/500	1/500	+1/500	1/100
		1752	1/100	+1/100 −1/20		
	2 doses of 0.5 cc.	1733	+1/500 −1/100	1/100	+1/500	−1/100
		1731	1/500	−1/100		

TABLE IV

Comparison of the Immunity Induced in Guinea Pigs by Different Antigens as Shown by Intracutaneous Tests

Antigen	Method of immunization	Animal No.	Fraction of M.L.D.			
			1/25	1/10	1/5	1/2
Refined toxoid No. 5-R	1 cc. in 1 injection	1739	±	±	Necrosis	Necrosis
		1736	−	−	±	Sl. redness −
	2 doses of 0.5 cc.	1711	±	±	Sl. necrosis	Necrosis
		1718	−	±	±	Necrosis
Veal infusion toxoid No. 25	1 cc. in 1 injection	1743	−	±	Redness 1 x 1½ cm.	Sl. necrosis
		1742	−	±	Redness 1 x 1½ cm.	Necrosis
	2 doses of 0.5 cc.	1724	−	−	±	Sl. redness +
		1721	−	±	±	Sl. redness
Infusion-free toxoid No. 5	1 cc. in 1 injection	1746	±	Necrosis	Necrosis	Necrosis
		1747	−	−	±	Sl. redness +
	2 doses of 0.5 cc.	1728	−	±	±	Necrosis
		1729	−	±	±	Sl. redness +
Toxin-antitoxin mixture No. 670	1 cc. in 1 injection	1751	±	±	Necrosis	Necrosis
		1752	−	−	±	Sl. redness
	2 doses of 0.5 cc.	1733	±	±	Sl. necrosis	Necrosis
		1731	±	Necrosis	Marked necrosis	Marked necrosis

The degree of immunity induced is indicated in the table in the following range: −, no reaction; ±, very slight reaction; slight redness −; slight redness; slight redness +; redness; slight necrosis; necrosis; marked necrosis.

The toxoids in these experiments proved to be more effective antigens than the toxin-antitoxin mixture. The immunity induced by two doses appeared to be similar to that obtained with one dose, as indicated both by the titration of the antitoxin in the blood and by the intracutaneous injection of standard toxin. The end-points of these titrations were not accurately determined because the variation of individual guinea pigs in their susceptibility or capacity to react was so great that further titration seemed scarcely worth while.

When the results of all the tests are reviewed, it is apparent that

the unconcentrated toxoids, No. 5 and No. 25, with equivalent flocculating values, were practically equivalent antigens. The refined toxoid (No. 5-R) gave rise only to slightly more antitoxin when the titration was made at the end of 4 weeks, but 8 weeks after the injection of two doses a larger number of units was obtained with this product.

The intracutaneous injection of standard toxin revealed no greater immunity with the refined than with the unconcentrated toxoid, despite the fact that the refined product had a definitely higher flocculating value than the original material, and that these tests were made 10 weeks after the last injection. In the process of refinement, the total volume of the final product is reduced to one-half that of the original material and, with the elimination of the nitrogen, there is a loss of antigenic value, estimated in terms of flocculation, which may be greater or less than 15 per cent. This product was highly refined and the loss evidently greater on that account.

The results of these intracutaneous tests of the immunity induced by one and by two doses of the toxoids correspond in one instance to the flocculating value obtained with toxoids No. 25 and No. 5 but do not parallel the flocculating value obtained with the refined product No. 5-R.

A comparison of the average units of antitoxin obtained with each antigen indicates that the refined product was uniformly and definitely the most effective antigen. The full extent of the immunity may not be accurately indicated either by the intracutaneous test, or by the estimation of antitoxin in the blood. It is not only conceivable that the several activities of toxin or toxoid which give rise to antitoxin in the blood may differ from those that induce the reaction in the skin after intracutaneous injection, but there are experimental observations indicating real differences (16). The variation in the response of the individual animals obscures the precise significance of a comparison of the results. Ramon (17) after an extended experimental study, accepts flocculation as the only true criterion of the antigenic value. Flocculating values appear to correspond, usually within a range of 10 to 20 per cent, with the standard titrations of antitoxin against the L_+ dose in the guinea pig and the flocculation test has become such a simple and helpful guide (18) that it has been, possibly, too generally accepted as of equivalent value in the standardization

of toxoid, but the exact relationship between the flocculating values and the antigenic activities in the tissues should be very accurately determined, especially with any new preparation of toxoid. Methods suggested by Bächer, Kraus, and Löwenstein (19), and more recently by H. Schmidt (20, 21), by which the affinity of toxoid for antitoxin can be titrated against an L_+ dose of standard toxin by subcutaneous injection in the guinea pig, or by intracutaneous injection of fractional amounts in quantitative relation, may provide more accurate means of titrating the antigenic activity of different toxoids.

The dosage of an antigen and the interval between the injections are such important factors in the development of the immune reaction that, unless and until the optimum conditions, in this respect, have been determined, it is difficult adequately to measure the effectiveness of an antigen in inducing immunity. The results of our tests may, therefore, fail to indicate the full value of these products as immunizing agents. Hence, the following experiments were undertaken with methods which have been more generally accepted in the standardization of such preparations.

Toxoid No. I, prepared from infusion-free diphtheria toxin broth by the addition of 0.28 per cent of formalin, had a flocculating value of 7.2 in 112 minutes. This toxoid was concentrated and diluted to half of its original volume. The flocculating value was then found to be 12.0 in 84 minutes. Its antigenic value as an immunizing agent in the guinea pig was determined by means of an intracutaneous test (22) and also by a subcutaneous test of immunity as required of manufacturers by the National Institute of Health, as follows:

> One series of guinea pigs was given immunizing doses of 0.5 cc. of the unconcentrated toxoid; another series was given the same amount of the refined toxoid. At the end of 3 weeks the immunity of all the guinea pigs was determined by the intracutaneous test—1/50 M.L.D. of standard toxin. If not immune they were retested at 7 day intervals until negative to this test.
>
> Another series of five guinea pigs was immunized with 0.5 cc. of the original toxoid No. I, and still another series with the refined and concentrated product No. I-R. After 4 weeks, both series of animals were tested with varying lethal doses of standard diphtheria toxin injected subcutaneously.

As compared with the previous studies, the results of both of these tests, which are recorded in Table V, indicate more definitely the ex-

tent of the immunization obtained with the purified toxoid. According to the immunity index it was two and one-half times as effective as the unconcentrated material. The results of subcutaneous inoculation of doses of 5, 10, 15, and 20 M.L.D. indicate a much greater degree of immunity. The results of these experiments and those of

TABLE V

Results of the Tests for Immunity with Intracutaneous and Subcutaneous Injections of Standard Toxin

Immunizing dose	Intracutaneous test		Subcutaneous test*		
	Immunity index†		M.L.D.	Animals surviving	
	Unconcentrated toxoid No. I	Refined toxoid No. I-R		Unconcentrated toxoid No. I	Refined toxoid No. I-R
cc.				*per cent*	*per cent*
0.5	2	1	5	60	100
0.5	2	1	10	60	100
0.5	2	1	15	20	60
0.5			20	0	80
0.2		1			
0.2		1			

Unconcentrated toxoid No. I had an Lf of 7.2; refined toxoid No. I-R an Lf of 12.0.

* The subcutaneous test for immunity formerly recommended by the National Institute of Health required that 80 per cent of the immunized guinea pigs survive 5 M.L.D. of standard toxin given 30 days after injection of the antigen. The test now recommended requires that 80 per cent survive 5 M.L.D. of standard toxin given 6 weeks after injection of the antigen. Five animals were used in each test.

† The immunity of the test animals is determined according to the method of Glenny (22); *i.e.*, weekly intracutaneous injections of 1/50 M.L.D. of standard toxin are given beginning 3 weeks after the immunizing dose. Absence of a reaction at the end of the 3rd week is designated as an "immunity index" of 1, at the end of the 4th week as 2, etc.

the flocculation test are not proportional. The difference in flocculating value, and in the time of flocculation, obtained with these two products does not correspond to the difference in immunizing action in the guinea pig. Variation in the flocculating values and the time observed in different tests, and the variation in the susceptibility of the guinea pig, may account for some, but not for all, of this discrepancy.

Flocculation is a helpful guide in the preparation of toxoid but the final standardization requires also tests of toxicity and of immunity in a susceptible animal. Moreover, when any new preparation is under investigation the practical results of immunization must be compared with those that follow immunization by the accepted practice, as, for example, the use of toxin-antitoxin mixture. More especially is this true since the character of the antigenic action of toxins or other bacterial products may be altered by the chemical processes used in refining or concentrating the product.

SUMMARY

By precipitation with acetone at 4°C. a refined diphtheria toxoid was obtained as a dry powder, readily soluble in aqueous solutions. The powder itself appears to be stable. When dissolved in half the original volume of physiological salt solution, the toxoid remained stable, in the cold room, for a period of 7 months. Only toxoids should be used which have been completely detoxified by the treatment with formalin. The formalin is immediately and completely removed by precipitation with acetone.

Filtration through a Mandler filter or the addition of a preservative did not appear to have any deleterious effect.

Incubating at 39°C. and freezing for 20 hours did not impair its activities.

The flocculating value of the purified toxoid containing phenol was almost completely destroyed by freezing, while when merthiolate was the preservative, it was unchanged.

Approximately 62 per cent of the nitrogen was eliminated in the process with approximately 15 per cent loss in flocculating value.

The flocculating values were nearly double those of the original material and thus corresponded with the concentration, which was one-half the volume. By this method, the antigenic activity of a toxoid below standard may be increased to a satisfactory standard. The antigenic activity of the refined product in the immunization of guinea pigs may in general parallel its flocculating values but the relation between the results of these tests may not be proportional nor constant. As an immunizing agent in the guinea pig, the refined toxoid

possesses an antigenic activity that is equal to, or greater than, that of the unconcentrated toxoid.

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