A QUANTITATIVE STUDY OF THE SCARLET FEVER TOXIN-ANTITOXIN FLOCCULATION REACTION*

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Since the discovery of scarlet fever toxin (Dick and Dick (1); Dochez (2)) there have been numerous attempts to isolate the active substance from the culture filtrates of scarlatinal strains of hemolytic streptococcus. Although the erythrogenic toxin has been purified to varying degrees, there has been little agreement in the literature as to its chemical nature or regarding the extent of purification attained. Some of the earlier workers suggested that the toxin might be a nitrogenous carbohydrate (Korschun *et al.* (3); Stock (4)). Dick and Boor (5) isolated a fraction of reduced nitrogen content containing 30,000 skin test doses per mg. The most active preparation of scarlet fever toxin that has been reported was that isolated by Stock (6). His purest material contained 11 per cent nitrogen and 10,000,000 skin test doses per mg. Stock's purest preparations presumably consist mainly of protein, but little has been stated regarding their chemical properties. On the other hand Barron, Dick, and Lyman (7) have recently carried out an extensive chemical study on material containing only 30,000 S.T.D.[1] per mg., which, from electrophoresis data, they regard as about 35 per cent pure. The latter authors conclude that scarlet fever toxin is a protein of low molecular weight and unusual stability. Finally, Koerber and Bunney (8) have described a "protein-free" scarlet fever toxin which they isolated from a casein hydrolysate medium. Their preparations contain only 0.7 to 1.0 per cent nitrogen and about 100,000 S.T.D. per mg. For further references to work on the purification of scarlet fever toxin, the paper by Stock (6) should be consulted.

In view of the contradictory results obtained by other workers, we have deemed it important to find some independent method for estimating the activity of pure scarlet fever toxin before actually attempting to isolate it in its purest form. The flocculation reaction appeared to offer possibilities for such a method. In a quantitative study of the Ramon diphtheria flocculation reaction, Pappenheimer and Robinson (9) showed that, within the equivalence zone, the nitrogen precipitated from a given amount of antitoxin varied linearly with the toxin added. The slope of this straight line was shown to represent

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[1] The abbreviation S.T.D. for skin test doses will be used throughout the rest of this paper.

the nitrogen of pure diphtheria toxin per Lf unit. Moreover, the method was found independent of the purity of the toxin and antitoxin preparations employed. Rane and Wyman (10) have described a flocculation reaction between scarlet fever toxin and antitoxin which they regard as a specific antigen-antibody reaction because of the close *in vivo-in vitro* correlation which they obtained using a great many preparations of toxin and antitoxin. If this flocculation reaction is indeed specific, then it should be possible to estimate the nitrogen per flocculating unit of pure scarlet fever toxin from a quantitative study of its reaction with antitoxin, providing that the reaction is analogous to the diphtheria system. Unfortunately, Evans and Gottschall (11) and Bunney and Koerber (12) have failed to obtain satisfactory correlation between the flocculation reaction and *in vivo* titrations of scarlet fever toxin, and the latter workers have expressed some doubt regarding the specificity of the reaction. It is hardly necessary to point out that before the flocculation reaction can be used to estimate the activity of pure scarlet fever toxin, its specificity must be established beyond reasonable doubt. With these factors in mind we have undertaken a quantitative study of the flocculation reaction of Rane and Wyman (10) between scarlet fever toxin and antitoxin.

EXPERIMENTAL

Cultures.—Toxins were prepared using two different scarlatinal strains of hemolytic streptococcus. Strain NY No. 5 has been most commonly used commercially for the production of scarlet fever toxin. It has been shown by Rane and Wyman (13) that strain No. 594 produces several times as much toxin as NY No. 5. Evidence that the toxin produced by No. 594 is identical with that of NY No. 5 has been given by Rane and Wyman, and by Plummer and Fraser (14). We are indebted to Dr. Leo Rane of the Antitoxin and Vaccine Laboratory, Jamaica Plain, Massachusetts, for cultures of the two strains.

Preparation of Toxin.—60 liters of culture of strain No. 594 and 30 liters of NY No. 5 were grown in 5 gal. pyrex carboys on the casein hydrolysate medium described elsewhere (15). After 60 hours growth at 35°C. with neutralization and continuous stirring the cultures were centrifuged and the supernate sterilized by Seitz filtration. The average yield of erythrogenic toxin from No. 594 was 9 Lf/cc. (*ca.* 300,000 S.T.D. per cc.), and from NY No. 5 was 2 Lf/cc. (*ca.* 60,000 S.T.D. per cc.).

Concentration and Partial Purification of Toxin.—The toxic filtrates from the two strains were concentrated and purified separately by the same procedure as follows: The culture filtrate was concentrated *in vacuo* at 30–35°C. to about 1/7 its original volume. The active material was then precipitated by adding 500 gm. of ammonium sulfate for each liter of concentrate and enough ammonia to keep the solution between pH 6.5 and 7.0. On standing overnight at room temperature, the toxin formed a dark flocculent layer near the surface. A large amount of crystalline sediment also appeared. The precipitates were filtered by suction and dissolved by dialysis against running water. After thorough dialysis the solution remained turbid and contained some precipitate which was removed by filtration through hard paper and discarded.

The volume at this point was 2 to 5 per cent that of the original culture filtrate. Ammonium sulfate was added to four-tenths saturation and a small amount of dark brown inactive precipitate was removed by filtration. This filtrate was brought to 0.75 saturation with ammonium sulfate and the toxin precipitated. The dark brown precipitate containing the toxin was separated by filtration through paper and dissolved in a small volume of water. The solution was dialysed against changes of distilled water and finally against Sørenson's phosphate buffer at pH 6.8 containing 1:5000 merthiolate. The flocculation reaction was used at each step to follow the fractionation of the toxin. No attempt was made to effect further purification.

Properties of Partially Purified Toxin.—From 60 liters of No. 594 culture filtrate, 400 cc. of purified toxin containing 950 Lf/cc., 3×10^7 S.T.D./cc., and 0.895 mg. nitrogen per cc. were obtained at this point, representing an overall yield of about 70 per cent. From 30 liters of NY 5, 33 cc. of purified toxin

TABLE I

Some Properties of Partially Purified Scarlet Fever Toxin

	Strain No. 594	Strain NY 5
Nitrogen content (per cent)	14.0	12.5
Activity (skin test doses per mg.)		
Rabbit	8,000,000	12,000,000
Human	8,000,000	12,000,000
Mg. nitrogen per Lf unit	0.00054	0.00035
Flocculation time for 38 Lf per cc. at 48°C. using antitoxin 531B (min.)	30	30
Per cent precipitable*	43	66

* Calculated using 0.00023 mg. nitrogen per Lf unit of toxin and based on the total nitrogen in the toxin.

were obtained, containing 800 Lf/cc., 2×10^7 S.T.D./cc., and 0.345 mg. nitrogen per cc., a yield of about 45 per cent. Both preparations gave strongly positive biuret, Millon and xanthroproteic tests in 0.3 per cent solution. Both preparations contained some material which was coagulated by heating to 100°C. Dilutions of the 594 toxin were injected intravenously into a few rabbits. These must be regarded as only preliminary experiments but have indicated that the M.L.D. for 2 to 3 kilo Chinchilla rabbits is about 50 Lf (1,500,000 S.T.D.) by the intravenous route. One rabbit given 95 Lf and 300 units of antitoxin survived. Rane and Wyman (13) report 180 Lf lethal for 5 lb. Chinchilla rabbits when injected subcutaneously. Some properties of the partially purified toxin after further dialysis are summarized in Table I.

In agreement with other workers (Kodama (16), Hooker and Follensby (17), and Barron *et al.* (7)) we have found the toxin to be relatively resistant to the action of the proteolytic enzymes, pepsin, papain, and trypsin. The flocculating power of the toxin was *not* impaired by treatment with these enzymes.

That these toxin solutions contained other streptococcal proteins was shown by a positive ring test when a 1/100 dilution of the concentrated toxin was added to an immune rabbit serum prepared against a smooth strain of hemolytic streptococcus.

Antitoxins.[2]—Antitoxin No. 2101 (Squibb Laboratories) was whole horse serum. Antitoxin No. 24739, also obtained from Squibb Laboratories, was a commercial preparation of pseudoglobulin. Antitoxins No. 531A and 531B, obtained from the National Drug Company, were from different bleedings from the same horse. They were solutions of the water-soluble globulin precipitated between 0.3 and 0.5 saturation with ammonium sulfate. Antitoxin No. 47E (National Drug Company) was a sample of enzyme-digested antitoxic pseudoglobulin. Antitoxin No. S113A was a pseudoglobulin preparation received from the Antitoxin and Vaccine Laboratory, Jamaica Plain, Massachusetts. The flocculation times of the various antitoxins varied between 25 and 130 minutes at 48°C. with 38 Lf units of toxin (See Table III).

The Flocculation Reaction.—The flocculation tests were done in the usual manner at 48°C. with a constant amount of toxin and increasing amounts of antitoxin. In evaluating flocculation titers or Lf units per cc. we have used the arbitrary standard set up by Rane and Wyman (10). In the quantitative studies, the amount of antitoxin in each tube was kept constant at 600 units. Increasing amounts of toxin were added and the volume in each tube was adjusted to 5 cc. with saline. These mixtures were set in a water bath at 38°C. for 3 hours and overnight in the cold. The tubes were then centrifuged, the supernates drawn off for test, and the precipitates broken up and washed three times with chilled saline. Nitrogen in the precipitates was determined by the micro-Kjeldahl method. The supernates were tested for excess toxin by skin tests in rabbits. All tests were carried out in duplicate.

Skin Tests.—The skin reactivity of toxin solutions and the supernates from the flocculation tests was determined by injecting graded dilutions into the skin of white or grey Chinchilla rabbits. The dilutions were made in saline in such a manner that each successive tube in a series contained half as much toxin as the preceding tube. The reactions after 24 hours and 40 hours were compared with similar reactions produced in the same rabbits by the Standard National Institute of Health toxin.[3] Three rabbits were used for each series of titrations and the results averaged. In the discussion of our results we have regarded the skin test values as significant only within ±50 per cent.

The skin reactivity of the concentrated and purified toxin preparations was confirmed *exactly* by tests carried out on susceptible human subjects using the Standard National Institute of Health toxin for comparison. We are greatly indebted to Dr. George F. Leonard of the Biological Laboratories, E. R. Squibb and Sons, for carrying out the human skin tests.

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[2] We are greatly indebted to Dr. W. E. Bunney of the Squibb Laboratories, New Brunswick, New Jersey, to Mr. A. M. Slee of the National Drug Company, Swiftwater, Pennsylvania, and to Dr. E. S. Robinson of the Antitoxin and Vaccine Laboratory, Jamaica Plain, Massachusetts, for the antitoxins used in this work.

[3] This toxin was supplied by Dr. M. V. Veldee of the National Institute of Health. It was labeled 30,000 S.T.D. per cc.

TABLE II

Quantitative Flocculation of Antitoxic Pseudoglobulin 531

Increasing amounts of scarlet fever toxin added to 600 units of antitoxin

I	II	III	IV	V	VI	VII	VIII
Toxin added	Toxin* nitrogen	Nitrogen† in precipitate	Antitoxin nitrogen in precipitate III-II	Ratio‡ A-nitrogen / T-nitrogen IV ÷ II	Total skin test doses added	Total skin test doses§ in supernate	Strain No.
Lf	*mg.*	*mg.*					
300	0.069	0.174			9,000,000	5,000,000	594
340	0.078	0.400			10,000,000	2,000,000	NY5
350	0.081	0.502			10,500,000		594
400	0.092	0.616‖			12,000,000		594
440¶	0.100	0.625			13,000,000	1,000,000	NY5
450¶	0.103	0.655			13,500,000	550,000	594
500	0.115	0.675	0.560	4.9	15,000,000		594
540	0.124	0.680	0.556	4.5	16,000,000	150,000	NY5
600	0.138	0.700	0.562	4.1	18,000,000	150,000	594
640	0.147	0.705	0.558	3.8	19,000,000	100,000	NY5
700	0.161	0.719	0.558	3.5	21,000,000		594
740¶	0.164	0.715			22,000,000	100,000	NY5
800¶	0.184	0.710			24,000,000	3,000,000	594
840	0.193	0.666			25,000,000		NY5
900	0.207	0.513			27,000,000		594
940	0.216	0.162			28,000,000	15,000,000	NY5
1000	0.230	0.224			30,000,000		594
600	0.138	—	anti-Ea**	—	18,000,000	7,500,000††	594
600	0.138	1.340	Ea-anti-Ea	—	18,000,000	5,000,000††	594
—	—	1.360	Ea-anti-Ea	—	—	none	594

* The nitrogen precipitated by 500 Lf units of toxin (column III) subtracted from that precipitated by 700 Lf and divided by 200 gives 0.00022 mg. nitrogen per Lf unit of toxin. The figure used in column II, however, is 0.00023 mg. of nitrogen per Lf unit, the average obtained from three titrations including the above.

† All values except the one marked ‖ are the averages of duplicate determinations.

‡ Values are calculated only for points of complete precipitation in the equivalence zone.

§ These values were determined in the skin of rabbits. They represent averages of tests on three rabbits.

¶ Incomplete flocculation.

** Ea = egg albumin.

†† These two values are undoubtedly low. They are included because they were determined on the same rabbits and show that there is no significant amount of toxin adsorbed to the egg albumin-anti-egg albumin precipitate.

RESULTS

The results of titrations of antitoxin 531 with both NY5 and 594 scarlet fever toxins are given in detail in Table II and plotted in Fig. 1. A quantitative diphtheria toxin-antitoxin titration is also plotted in Fig. 1 for comparison.

The reaction between scarlatinal toxin and antitoxin resembles the quantitative diphtheria toxin-antitoxin reaction in several respects. Thus, for example, precipitation occurs within a narrow zone only. Moreover, the Danysz phenomenon is readily demonstrable by flocculation in the inhibition zone of antitoxin excess and although not indicated in Table II, its magnitude is the same as with the diphtheria system. Thus after 200 Lf of scarlet fever toxin were incubated with 600 units of antitoxin, no flocculation occurred. However,

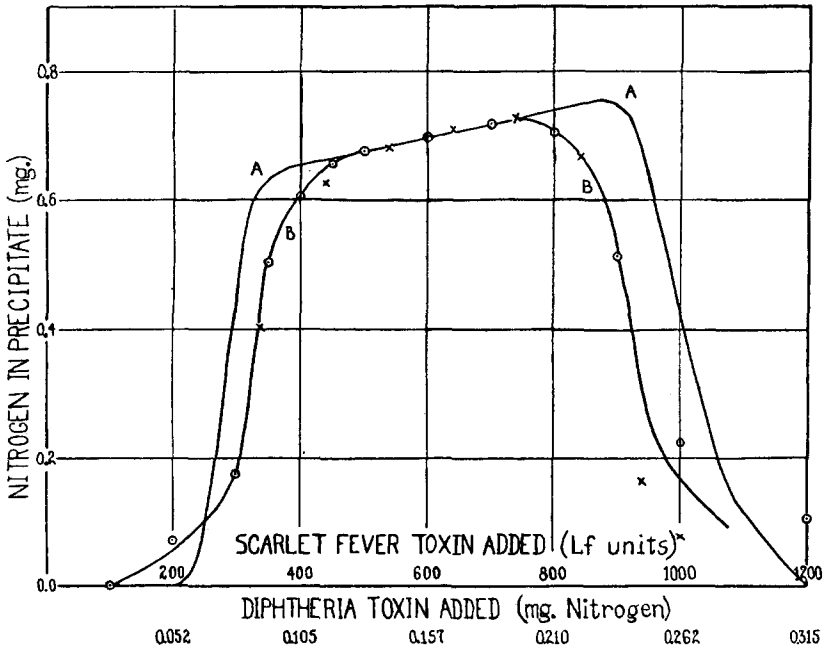

FIG. 1. Quantitative toxin-antitoxin flocculation reaction. Curve A, diphtheria system, Pappenheimer and Robinson (9). Curve B, scarlet fever system. ⊙ = 594 toxin. x = NY5 toxin.

when the mixture was then treated with increasing amounts of toxin, only 200 units of antitoxin were found by flocculation rather than 400 units expected if there were no Danysz phenomenon. In spite of these similarities, certain important quantitative differences exist between the diphtheria and scarlet fever toxin-antitoxin reactions. Although the width of the flocculation zone is almost exactly the same for both reactions, the region of nearly *complete* precipitation is more than twice as broad for the diphtheria toxin-antitoxin system as for the scarlet fever toxin-antitoxin system. This is not only true for antitoxin No. 531 shown in Fig. 1, but holds for all the scarlet fever antitoxins which we have studied. In fact antitoxin No. 531 was chosen for

detailed study because its zone of nearly complete precipitation was the broadest of all the scarlet fever antitoxins we have examined.

Several quantitative titrations with various antitoxin preparations are summarized in Table III.

TABLE III

Results from Various Toxin-Antitoxin Titrations

I	II	III	IV	V	VI	VII	VIII
Antoxin No.	Units antitoxin per cc. by flocculation	Flocculation time (38 Lf/cc. at 48°C.)	Toxin strain No.	Toxin nitrogen per Lf	Maximum specifically* precipitable antitoxin nitrogen from 600 units antitoxin	Total nitrogen specifically precipitated	Ratio $\frac{\text{A-nitrogen}}{\text{T-nitrogen}}$ at flocculation point
		min.			*mg.*	*per cent*	
2101	133	25	594	(0.00036)†	0.581	1.0	4.2
531A	315	35	594	0.00022	0.560	1.9	4.1
531B	250	30	NY5	0.00021	0.552	2.0	4.0
S113A	940	130	594	—‡	0.612	3.9	4.4
24739	880	110	594	0.00024	0.484	2.4	3.5
47E	720	46	594	—‡	0.458	1.9	3.3

* Calculated using 0.00023 mg. nitrogen per Lf unit of toxin.

† The results of this titration were irregularly high, indicating that the precipitates were not washed sufficiently.

‡ The equivalence zone was too narrow to calculate a value.

DISCUSSION

In the previous study of the diphtheria flocculation reaction (9), it was assumed that throughout the equivalence zone precipitation was essentially complete, and that even if the specific toxin-antitoxin complex were slightly soluble within the zone, no appreciable dissociation occurred, since no toxin could be detected in the supernate by the sensitive rabbit intracutaneous test. This is not true in the present instance. Even at the flocculation point the scarlet fever toxin-antitoxin complex is somewhat soluble and the soluble complex is apparently dissociated to an appreciable extent. Since it is practically completely dissociated at high dilutions, the skin test method may be used to determine roughly its solubility. Column III of Table IV shows that the solubility of the toxin-antitoxin complex at the flocculation point remains practically constant over a 50-fold range of concentration. The solubility at ice box temperature corresponds to about 1 Lf per cc. (30,000 S.T.D. per cc.).

It is well established that antigen-antibody reactions are reversible and that under certain conditions the antigen-antibody complex may dissociate. Glenny, Pope, and Waddington (18) have presented evidence that the diphtheria toxin-antitoxin complex may dissociate to an appreciable extent upon dilution, particularly when antitoxins of low "avidity" are used. When dilute

solutions of diphtheria toxin are neutralized *in vivo*, relatively more antitoxin of low "avidity" is necessary for complete neutralization than would be predicted from *in vitro* tests carried out using more concentrated solutions. In our opinion the dissociation of the toxin-antitoxin complex upon dilution provides a reasonable explanation for the large discrepancy between the *in vitro* and *in vivo* neutralization of scarlet fever toxin by antitoxin noted by Rane and Wyman (10). Rane and Wyman found 60,000 S.T.D. neutralized per unit of antitoxin in the flocculation test rather than the expected *in vivo* neutralization of only 50 S.T.D. In the latter case toxin and antitoxin are present in fairly high dilution. If the dissociation constants are large, it follows from the

TABLE IV

Skin Tests on Supernates from Floccules at the Flocculation Point

I Toxin and antitoxin concentration at flocculation point	II Skin test doses per cc. of mixture	III Skin test doses* found in supernate per cc.	IV Total toxin-antitoxin complex found in supernate at flocculation point
units per cc.			*per cent*
10	300,000	30,000	10.0
40	1,200,000	30,000	3.0
60	1,800,000	20,000	1.0
120	3,600,000	40,000	1.0
180	5,400,000	40,000	0.7
475	13,000,000	80,000	0.6

All mixtures were set at 38°C. for 3 hrs., then at 6°C. for 18 hrs.

* These values were determined in the skin of rabbits. They represent averages of tests on three rabbits.

A curious observation was made during these tests. Although the reactions after 24 hours were similar, after 4 days those from the supernates with 180 units or less per cc. had faded, but those from the 475 unit supernate persisted with increased intensity. There was no increase in size.

mass law that an excess of antitoxin must be used if neutralization is to be complete. In the flocculation reaction last traces of toxin need not be neutralized. We have found 30,000 S.T.D. neutralized by one unit of antitoxin in the flocculation test but do not regard this difference from 60,000 as particularly significant in view of the difficulties of skin testing in rabbits.

As we have already pointed out in the introduction, before the flocculation reaction can be used for predicting the potency of pure scarlet fever toxin and antitoxin, it is necessary to prove that the reaction is a specific one between the toxin itself and antitoxin. From an examination of Table II it will be noted that within the region of maximal precipitation (*i.e.* 500 to 740 Lf toxin with 600 units of antitoxin) less than 1 per cent of the toxin was found in the supernatant by intracutaneous rabbit test. Outside the equivalence zone, no

apparent neutralization occurs when tested by the intracutaneous method, presumably because of dissociation. That the precipitation of the toxin is not due to non-specific absorption by the precipitate is indicated by the fact that even twice as much specific egg albumin-anti-egg albumin horse antibody precipitate formed in the presence of scarlet fever toxin, failed to carry down the toxin (Table II). We feel that these demonstrations of the specific precipitation of scarlet fever toxin by antitoxin in the equivalence zone are conclusive evidence that the reaction we are dealing with is in fact specific.

Evans and Gottschall (11) and Bunney and Koerber (12) have recently reported failure to obtain adequate correlation between the flocculation titer and the *in vivo* potency of scarlet fever toxins. It seems worth while to discuss possible reasons for the discrepancy in view of the conclusion drawn above. The fact that the present reaction is specific is not proof of its usefulness as a general method of assay, and does not mean that other flocculating antibodies do not occur in antitoxins made by immunizing with scarlet fever toxins produced on infusion and peptone media. Rane and Wyman (10) note that occasional serums showed double zones with certain toxins, of which only one zone appeared to be specific. Bunney and Koerber (12) found that concentrates of infusion broth or peptone gave flocculation reactions with certain scarlet fever antitoxins. We have confirmed the observation of Bunney and Koerber that flocculation may occur with peptone and antitoxic horse serum. However, after removal of the non-specific antibody by flocculation with concentrated peptone solution, the antitoxin still flocculated to titer with scarlet fever toxin. Bunney and Koerber also reported that they were unable to flocculate toxin produced on a casein hydrolysate medium. Using a particularly rapidly flocculating antitoxin we were able to flocculate some of this casein hydrolysate toxin kindly sent us by Dr. Bunney and Dr. Koerber. The toxin which they sent us contained 200,000 S.T.D. per cc. and flocculated slowly at 7 Lf per cc.

While the non-specific reactions discussed above may account for some of the failures to obtain satisfactory *in vivo-in vitro* correlation, it seems unlikely that this can be the whole explanation of Evans and Gottschall's results since they tested their toxins over a wide range and report only one zone of flocculation. There is another possible explanation for the observed discrepancies in titrating scarlet fever toxins. Hooker and Follensby (17) have demonstrated that certain scarlet fever strains of streptococcus, including the NY5 strain, may under certain conditions, produce two erythrogenic toxins, A and B. The B toxin is more labile than and immunologically distinct from the ordinary Dick toxin. The presence of two immunologically dissimilar skin toxins would, of course, interfere with their assay by the flocculation test. In our own experience, we have encountered no difficulty in following the purification of toxin from both NY5 and No. 594 strains by means of the flocculation reaction.

Presumably, under our conditions for growing the organisms, no appreciable amount of the Toxin B of Hooker and Follensby was produced.

The studies of Rane and Wyman (13) and Plummer and Fraser (14) have suggested that the toxin produced by strain No. 594 is identical in its immunological behavior to NY5 toxin. The former has not been accepted as yet by the Scarlet Fever Committee. Table II and Fig. 1 show that the No. 594 and NY5 toxins are quantitatively identical in the flocculation reaction within the limits of experimental error. Because the No. 594 strain produces several times as much toxin as the NY5 strain under similar conditions, the former strain should prove useful for toxin production.

Since skin tests on the supernatants from the equivalence zone show that at least 99 per cent of the toxin-antitoxin complex is precipitated from 600 units of antitoxin by 500 to 740 Lf of toxin, it is possible to calculate the nitrogen per Lf unit for pure scarlet fever toxin from the slope of the quantitative flocculation curve within this region. From three titrations on two different antitoxins, careful nitrogen determinations indicated that pure scarlet fever toxin contains 0.00023 mg. nitrogen per Lf unit, a value which we believe to be accurate to within at least 20 per cent. This nitrogen per Lf unit does not have the same significance as in the case of diphtheria toxin because there is no standard flocculating antitoxin available for comparison. It may also be calculated from the data in Tables II and III that pure scarlet fever toxin contains very close to 1.3×10^8 skin test doses per mg. of nitrogen. Similar calculations indicate that pure scarlet fever antitoxin contains 0.00093 mg. nitrogen per unit. Since the concentrated and partially purified No. 594 toxin contained 0.00054 mg. nitrogen per unit and 5.7×10^7 S.T.D. per mg. nitrogen, we estimate that the preparation contains about 43 per cent scarlet fever toxin assuming that the toxin has the same nitrogen content as protein. Similarly, the NY5 preparation contains about 66 per cent scarlet fever toxin.

From Table III it will be noted that with two exceptions the maximum antitoxin nitrogen precipitated by toxin from 600 units of antitoxin varied between 0.552 and 0.612 mg. The two exceptions are antitoxins No. 47E and No. 24739. Antitoxin No. 47E (National Drug Company) had been treated with pepsin according to the procedure of Pope (19) and from its low precipitable nitrogen it may be assumed that a splitting of the molecule has occurred analogous to pepsin-treated diphtheria antitoxin (9, 20). Antitoxin No. 24739 (Squibb Laboratories) was a concentrated pseudoglobulin preparation which had not been subjected to the action of pepsin. We are unable to explain its low specifically precipitable nitrogen content at this time.

According to the above calculations, most of the preparations of scarlet fever toxin which have been reported in the literature were of a very low order of purity. We feel that detailed chemical studies on such preparations should be viewed with some skepticism. Thus the recent work of Barron, Dick, and

Lyman (7) should be interpreted with caution, since according to our calculations, their preparation contained only about 0.2 per cent actual toxin. On the other hand, it seems likely that the preparations of Stock (6) which contained 10^8 S.T.D. per mg. of nitrogen, were of a very high degree of purity. In agreement with Stock (6) and Barron, Dick, and Lyman (7) our results suggest that scarlet fever toxin is a protein. Our preparations, estimated to be about 45 per cent and 66 per cent pure, contained 14 per cent and 12.5 per cent nitrogen respectively and gave strongly positive protein tests in dilute solution. We also feel that the striking qualitative resemblance of the scarlet fever toxin-antitoxin reaction to other protein-antiprotein flocculation reactions, in the horse, namely diphtheria toxin-antitoxin (9), egg albumin-anti-egg albumin (21), and hemocyanin-antihemocyanin (22), constitutes strong supporting evidence for the conclusion that scarlet fever toxin is a protein. In agreement with other workers we have found scarlet fever toxin to be a protein of unusual resistance to action of the proteolytic enzymes pepsin, trypsin, and papain.

The ratio of antitoxin nitrogen to toxin nitrogen at the flocculation point is 4.1:1 for the scarlet fever system (Tables II and III). This is somewhat higher than the corresponding ratio of 3.6:1 found for the diphtheria toxin-antitoxin complex, and in our opinion suggests that the nitrogen content of scarlet fever toxin is lower than that of diphtheria toxin. Both Stock's purest preparations (6) and our own contain only 11 to 14 per cent nitrogen. It therefore appears likely that the composition of the floccules at the flocculation point is almost exactly the same for the two systems. The *molecular* composition at the flocculation point for the diphtheria toxin-antitoxin and egg albumin-anti-egg albumin (horse) systems corresponds respectively to TA_2 and EaA_2 (21). If now we assume that scarlet fever antitoxin has the same molecular weight as diphtheria antitoxin (180,000) and that the molecular composition of the specific precipitate with toxin is the same for the two systems, then it may be predicted that the molecular weight of scarlet fever toxin will be found to be about the same as that of diphtheria toxin, *i.e.* about 70,000 (20, 23).

SUMMARY

1. Highly purified scarlet fever toxin has been prepared from culture filtrates of two scarlatinal strains (NY5 and 594B) of hemolytic streptococcus grown on a medium of defined composition.

2. The flocculation reaction of Rane and Wyman has been studied quantitatively and has been shown specific for scarlet fever toxin and antitoxin.

3. Scarlet fever toxin from strains NY5 and 594B are quantitatively identical in their immunological behavior.

4. Pure scarlet fever toxin contains 0.00023 mg. nitrogen per flocculating unit and close to 1.3×10^8 skin test doses per mg. nitrogen as calculated from

the immunological data. Both the immunological and the analytical data suggest that scarlet fever toxin is a protein. Similar calculations indicate that scarlet fever antitoxin contains 0.00093 mg. nitrogen per unit.

5. The scarlet fever toxin-antitoxin complex is readily dissociated in dilute solutions. In this respect the scarlet fever toxin-antitoxin system contrasts sharply with the diphtheria toxin-antitoxin system.

6. The scarlet fever toxin-antitoxin reaction is discussed in relation to other flocculation reactions.

Since this paper went to press further evidence for the specificity of the scarlet fever toxin-antitoxin flocculation reaction has appeared. H. Proom (*J. Path. and Bact.*, 1941, **53,** 39) has succeeded in recovering scarlet fever toxin in 5 to 10 per cent yield from washed toxin-antitoxin floccules by tryptic digestion.

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