STUDIES ON THE MOLECULAR WEIGHT OF DIPHTHERIA TOXIN, ANTITOXIN, AND THEIR REACTION PRODUCTS

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The toxin-antitoxin flocculation reaction has long been regarded as an atypical example of the precipitin reaction. Marrack (1) states, "The behavior of the diphtheria toxin-antitoxin system is altogether abnormal. The range within which precipitation occurs is very narrow and no precipitate forms when the ratio of antibody to antigen exceeds about twice the ratio at the equivalence point." In addition, antitoxin is associated with the pseudoglobulin fraction of immune horse serum in contrast to antibacterial antibodies which are associated with the water-insoluble or euglobulin fraction. It must be remembered, however, that practically all the work on protein-antiprotein systems has utilized antibody obtained from the rabbit. The only systems using horse antibody which have been extensively studied, excepting toxin-antitoxin reactions, have been polysaccharide-antipolysaccharide systems. In other words, the exceptional character of the toxin-antitoxin reaction may be merely a species difference in the antibody used and the reaction may well be a typical example of horse antiprotein systems. Evidence to suggest that this is indeed the case has been furnished by immunizing a horse against crystalline ovalbumin. As will be reported subsequently, the ovalbumin-antiovalbumin system in the horse gives rise to a reaction characteristic of diphtheria toxin-antitoxin flocculation. Moreover, diphtheria antitoxin produced in the rabbit gives a typical precipitin reaction.

During the last ten years considerable advance has been made in our knowledge of the nature of the reaction between antigen and antibody in immune systems. In particular, the introduction of absolute quantitative methods by Heidelberger and Kendall for determining the quantity of antibody entering into the precipitin reaction and the composition of the specific precipitates (2), has made it possible to develop a theory of antigen-antibody union on the basis of the law of mass action, assuming a series of

competing bimolecular reactions (3). The past few years have seen the general acceptance of the protein nature of antibodies and it is now generally assumed that antibodies are slightly "modified" serum globulins (1, 3). From the effect of high salt concentration on the quantitative reaction between pneumococcus polysaccharide and antipolysaccharide, it was found (4) that less antibody was combined at high ionic strength with a given amount of polysaccharide than in solutions of low ionic strength. On the basis of this finding, Heidelberger and Kendall (5), were able to dissociate the specific precipitate and obtain 100 per cent specifically precipitable antibody. Confirmation of the theory could then be obtained by an ultracentrifugal study of the components entering into the reaction (6). By studying the sedimentation behavior of specific ovalbumin-anti-ovalbumin dissolved in excess pure ovalbumin, empirical formulas for antigen-antibody complexes were calculated (7). Further work showed that pneumococcus antibodies vary greatly in different species (8). Thus pneumococcus antibody in the horse is concentrated in the euglobulin fraction and has been found to have a molecular weight of 990,000; s_{20} = 18×10^{-13} cm./sec./dyne. Although normal serum globulin preparations always contain a small amount of material having this sedimentation constant, this fraction is enormously increased in hyperimmunized horses. Moreover, electrophoresis studies by the new method of Tiselius (9) show that this high molecular weight component represents a new major globulin fraction of the immune serum. By contrast, rabbit antipneumococcus antibody has a sedimentation constant indistinguishable from that of normal rabbit globulin and cannot be separated by electrophoresis (10).

Since the diphtheria toxin-antitoxin reaction behaves so differently from the other systems just discussed, it has seemed worth while to investigate it by the methods utilized by Heidelberger and others for study of the precipitin reaction. A careful study of the nitrogen content of toxin-antitoxin floccules (11) together with the isolation of pure diphtheria toxin protein (12, 13) enabled us to calculate the ratio of antitoxin to toxin nitrogen throughout the equivalence zone. Toxin was found to be "multivalent" with respect to antitoxin, in agreement with results of other workers (Danysz, 14; Healey and Pinfield, 15; Eagle, 16; Pope and Healey, 17) and the composition of the specific floccules was found to vary approximately threefold throughout the equivalence zone. The present communication deals with the electrophoretic, sedimentation, and diffusion behavior of the two purified reactants and their reaction products in regions of toxin and antitoxin excess. In a preliminary report (Lundgren, Pappenheimer, and Williams, 18) diphtheria toxin was shown to be homogeneous by sedimenta-

tion and electrophoresis, and electrodialyzed antitoxic pseudoglobulin, 33 to 35 per cent specifically precipitable by toxin, was shown to be essentially homogeneous in the ultracentrifuge with a sedimentation constant close to that of the normal serum globulins.

EXPERIMENTAL

In order to study the reaction between toxin and antitoxin by sedimentation, electrophoresis, and diffusion, it was important first to analyze the behavior of the two purified reactants by these methods. In all experiments the Wiener-Lamm refractometric methods were used. Concentration of components were calculated from areas measured under the usual scale displacement distance curves.

Toxin.—The same highly purified diphtheria toxin which has been previously described (13) was studied in 1 per cent solution in $M/15$ phosphate buffer at pH 6.9, 0.172 molar in sodium chloride. Seven experiments in the standard Svedberg oil turbine ultracentrifuge gave an average sedimentation constant, $s_{20} = 4.73 \times 10^{-13}$ cm./sec./dyne. The curves by the scale displacement method appear very homogeneous and no significant trace of any other high molecular weight constituent was present. A provisional value for the diffusion constant was obtained with a 0.4 per cent solution of the toxic protein by observing the blurring of a boundary formed in one section of the Tiselius electrophoresis apparatus. The diffusion constant, corrected to a process taking place in pure water at 20°C. is close to $D_{20} = 6.2 \times 10^{-7}$ cm.^{2}/sec. Since the partial specific volume was found to be 0.736, the molecular weight of the toxic protein may be calculated from the formula

$$M = \frac{RTs}{D(1 - V\rho)} \cong 70,000$$

Using a 1 per cent solution of the toxin at pH 7.0 and ionic strength 0.1, the protein migrated essentially as a single component with a mobility of 4.9×10^{-5} cm.^{2}/sec./volt in the Tiselius apparatus. However on closing the schlieren diaphragm way down, a small amount of a faster component could be observed, probably due to a small amount of inactive bacterial protein (2 to 5 per cent) known to be present (13).

In conclusion, the toxin would appear to be essentially homogeneous by the methods used.

Antitoxin.[1]—The isolation of diphtheria antitoxin in immunologically pure form has not been accomplished by us as yet, although preparations of pseudoglobulin of high antitoxin content have been prepared which satisfy all the ordinary criteria for purity of a protein by the usual chemical and physical tests.

It seems unlikely at this time that the separation of antitoxic pseudoglobulin from associated inactive globulin can be effected by the usual methods of salting out and dialysis, as has been reasonably successful with the pneumococcus antibodies (Chow and Goebel, 19; Green *et al.* 20; Kendall, 21). In our experience, the potency of antitoxin which it is possible to prepare by fractionation of the serum globulins from any given antitoxic horse serum, is directly proportional to that serum's original purity. Thus serum 726, containing 525 units antitoxin per cc., after repeated fractionation with ammonium sulfate and dialysis, yielded a pseudoglobulin preparation only 9.6 per cent specifically precipitable by toxin; serum 1880 obtained from Squibb Laboratories, containing 1440 units per cc., yielded antitoxic pseudoglobulin which was 33 per cent precipitable and serum 675–676, obtained from the Connaught Laboratories, containing 2000 units per cc., readily gave a product which was 42 per cent precipitable. Further fractionation by salting out, electrodialysis, and isoelectric precipitation (Green *et al.*, 20) failed to increase materially the proportion of these pseudoglobulin preparations which could be specifically precipitated by toxin.

Attempts were next made to obtain pure antitoxin by dissociation of the specific precipitates formed with toxin. There are several reports in the literature of the successful dissociation of toxin-antitoxin floccules. Ramon (22), as early as 1923, succeeded in obtaining a 75 per cent recovery by dissolving the floccules in dilute acetic acid in the absence of salt and heating to 56–60°C. for one hour. This treatment destroyed the toxin, but had little effect on the antitoxin. Ramon's work was repeated in 1926 by Locke and Main (23); and later Glenny and Pope (24) and Modern and Wernicke (25) obtained antitoxin recovery without heating. In the latter case the yields were very low except in extremely dilute solution.

We have been unable to obtain appreciable dissociation of specific floccules in acid solution with the toxin-antitoxin preparations used without the aid of heat. We are at loss to explain our failure to repeat the previous work, but it may depend on the "avidity" of the particular serums used. We have also tried several other methods of dissociation.

Both high salt concentration and electrodialysis were without effect on the equilibrium. Diphtheria toxin is denatured by solution in 20 per cent urea and soon loses its ability to combine with antitoxin even after the urea has been removed by dialysis. On the other hand the activity of antitoxin is unaffected by strong urea solutions. Although it was possible to dissolve specific floccules in 20 per cent urea, no evidence for dissociation could be detected. Similarly dissociation with trypsin failed. Diphtheria toxin is very sensitive to the action of trypsin, whereas antitoxin loses activity only after prolonged treatment with the enzyme. However, combination of toxin with antitoxin appears to protect the former from tryptic digestion in the same way that it is protected from denaturation by acid and by urea. Diphtheria toxin has an isoelectric point at pH 4.1, whereas that of antitoxic pseudoglobulin is close to pH 6.0. It was thought that if any dissociation occurred in acid regions, it might be possible to separate the two proteins by electrophoresis at pH 4.5. Unfortunately this was not the case. A further experiment was carried out in acetate buffer, pH 3.6, using the Tiselius apparatus to follow the boundaries. The dissolved floccules showed nearly homogeneous

migration, except for a very faint trace of slow moving material. It was evident that no significant dissociation had occurred at this pH. Very recently, since these experiments were completed, Pope and Healey (26) have described dissociation by means of short treatment with pepsin at pH 3.0. They obtained 70 per cent recovery of material which apparently approached immunological purity. We have confirmed the fact that active antitoxin can be recovered by their procedure, but have had no opportunity to test the homogeneity of the dissociated material.

Preparation of Antitoxic Pseudoglobulin.—Antitoxin 1880 contained 1440 units per cc. 900 cc. of plasma were treated with 450 cc. distilled water and 1350 cc. saturated ammonium sulfate. After standing overnight in the cold, the precipitate was collected and dialyzed against running water for 2 days. The euglobulin precipitate was centrifuged and washed once with distilled water. The supernatant and washing, measuring 550 cc., were treated with 275 cc. saturated ammonium sulfate and left overnight in the cold. The precipitate was centrifuged, collected, and dissolved. After dialysis against running tap water for 48 hours followed by changes of distilled water and removal of precipitated euglobulin, this fraction contained a total of 233,000 units and 18.9 per cent of the nitrogen was specifically precipitable by toxin. The supernatant from this one-third saturated precipitate was brought to half saturation with ammonium sulfate. The precipitate was collected in the centrifuge and dialyzed as above. The pseudoglobulin fraction so obtained contained 800,000 units, or 61.5 per cent of the total antitoxin of the original plasma. 32.5 per cent of the nitrogen was specifically precipitable by toxin. After electrodialysis the precipitable nitrogen was raised to 33 to 35 per cent. Further attempts to purify the antitoxin by salting out and isoelectric fractionation were unsuccessful.

This electrodialyzed preparation of antitoxic pseudoglobulin showed a main component in the ultracentrifuge having a sedimentation constant $s_{20} = 7.4 \times 10^{-13}$. There was also present a small amount (about 5 per cent) of material with $s_{20} = 18 \times 10^{-13}$ and probably traces of other components as well, since somewhat imperfect base lines were obtained.

Electrophoresis of Antitoxic Pseudoglobulin.—Antitoxic pseudoglobulin, prepared as above, was run in the Tiselius apparatus in phosphate buffer at pH 7.35, ionic strength 0.1. Two components were observed by the schlieren method with mobilities 4.9×10^{-5} and 2.6×10^{-5} cm.^{2}/sec./volt. The faster moving boundary is undoubtedly identical with the α-globulin of Tiselius (9) whereas the slower moving material represents a new component, not described by Tiselius and probably only present in traces in normal horse pseudoglobulin according to our own observations. From the areas under the scale displacement-distance diagrams approximately 25 per cent of the material was α-globulin and the remaining 75 per cent the new component. It could readily be demonstrated that the antitoxin was associated with the new component, since the concentration of α-globulin remained unchanged in the toxin-absorbed inactive pseudoglobulin while the slower moving boundary was markedly diminished after removal of

the antitoxin. No bands corresponding to serum albumin, β-, or γ-globulin were observed.

For most of the sedimentation and diffusion experiments α-globulin-free antitoxic pseudoglobulin was used. This was prepared in the large Tiselius apparatus[2] by using 100 cc. of a 2 per cent solution of electrodialyzed antitoxic pseudoglobulin at pH 7.3, ionic strength 0.02. Owing to the high protein concentration and low ionic strength, boundary anomalies and often slight convection currents were observed. However, the increased mobility at the lower ionic strength more than outweighed these difficulties, particularly if a second separation was carried out on the same material. Antitoxic pseudoglobulin prepared in this way was 43.5 per cent specifically precipitable and showed a single band on electrophoresis with mobility

TABLE I

Some Physical Properties of Diphtheria Toxin and α-Globulin-Free Antitoxic Pseudoglobulin

	Toxin	Antitoxic pseudoglobulin
Specific volume	0.736	—
Sedimentation constant $\times 10^{13}$	4.7	7.2
Diffusion constant $\times 10^{7}$	6.2	4.40
Molecular weight	70,000	150,000*
Svedberg dissymmetry number f/f_0	1.2	1.4
Mobility $\times 10^5$ (pH 7.35 ionic strength 0.1)	4.9	2.6
Nitrogen content	16.0 per cent	14.3 per cent

* Assuming partial specific volume = 0.736.

2.6×10^{-5} cm.^{2}/sec./volt in phosphate buffer, pH 7.35, ionic strength 0.1. No trace of α-globulin or other components could be detected in the fractionated material.

A characteristic of the electrophoresis boundary of antitoxic pseudoglobulin at pH 7.3 is its inhomogeneity. The band tends to broaden and become diffuse with time in a symmetrical manner and even after several hours shows no tendency to split up into more than one component. In other words, the protein behaves as if there were several components present differing only slightly in charge. On reversal of the current, the band sharpens once more. After removal of the antitoxin by absorption with pure toxin, the remaining inactive pseudoglobulin has the identical mobility and shows the identical tendency to become far more diffuse with time

[2] We are indebted to Professor E. J. Cohn of The Harvard Medical School for permission to use the large Tiselius apparatus in his laboratory.

than can be accounted for by ordinary diffusion.[3] Thus it does not seem likely that this electrical inhomogeneity is due to any impurities present in the preparation.

When α-globulin-free pseudoglobulin was run in the ultracentrifuge, far better scale displacement diagrams were obtained than with the unfractionated material. The faster sedimenting component, $s_{20} = 18 \times 10^{-13}$, had entirely disappeared and the perfect base lines indicated that all the protein material present had a sedimentation constant, $s_{20} = 7.2 \times 10^{-13}$ cm./sec./dyne. Some of the physical properties of α-globulin-free antitoxic pseudoglobulin are summarized in Table I together with properties of diphtheria toxin.

Diffusion Constant of α-Globulin-Free Antitoxic Pseudoglobulin

Two determinations of the diffusion constant of the above antitoxic pseudoglobulin were made at concentrations of 0.4 and 0.8 per cent protein in phosphate-borate buffer at pH 6.9, and 25°C. The solutions were 0.172 molar in sodium chloride. The blurring of a boundary formed against the same buffer in a cell of the Lamm-Polsen (27) type was followed with time by the refractive index method. The average diffusion constants found in the two experiments were 4.38×10^{-7} and 4.42×10^{-7} cm.^{2}/sec. respectively, corrected to pure water at 20°C. Two of the scale displacement diagrams were analyzed by the method of moments, and the points were found to lie extremely closely upon an ideal distribution curve (Fig. 1). In one case, the standard deviation calculated from the moments was 0.286 and from the diffusion constant $\delta = \sqrt{2Dt} = 0.285$, where D is the diffusion constant and t the time in seconds. In the other case, the standard deviation by the two methods of calculation was 0.261 and 0.258 respectively. Assuming a value of 0.736 for the partial specific volume and taking $s = 7.2 \times 10^{-13}$ and $D = 4.40 \times 10^{-7}$ the molecular weight of antitoxic pseudoglobulin is calculated to be 150,000.

Absorption of Antitoxic Pseudoglobulin with Toxin.—12.8 cc. of α-globulin-free antitoxic pseudoglobulin in phosphate-borate buffer, pH 7.0, ionic strength 0.02, containing 1 per cent NaCl and 1:10,000 merthiolate as preservative, with 550 units of antitoxin per cc. were treated with the same number of Lf of pure toxin contained in 3.5 cc. of the same buffer. After flocculating overnight in the cold, the specific precipitate was centrifuged and washed twice with chilled saline and then once with distilled water.

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[3] Calculations by Mrs. M. Bruch-Willstätter have shown that this broadening cannot be due to free diffusion alone. We are grateful for this and other assistance rendered by her.

The supernatant solution of inactive pseudoglobulin was used as a control for sedimentation experiments. The washed precipitate was saved for analysis and the washings were discarded.
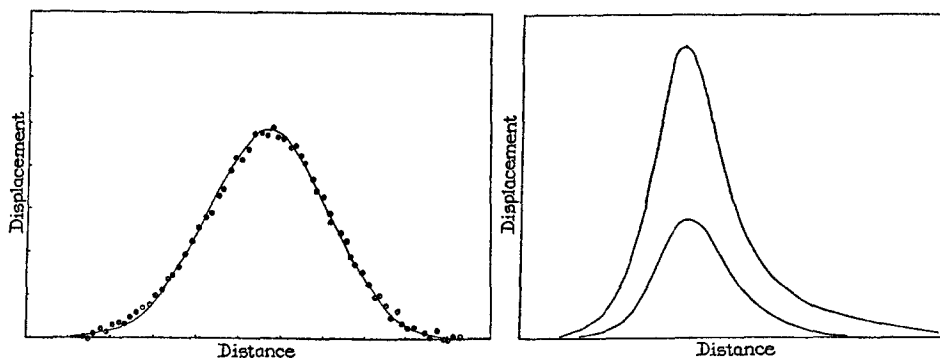

FIG. 1   FIG. 2

FIG. 1. Diffusion diagram of α-globulin-free antitoxic pseudoglobulin after 65,000 seconds. Heavy line is ideal distribution curve calculated by method of moments.

FIG. 2. Sedimentation diagram of antitoxic α-globulin-free pseudoglobulin before and after removal of antitoxin by specific precipitation with toxin.

TABLE II

Some Properties of Antitoxic Pseudoglobulin before and after Specific Absorption with Pure Diphtheria Toxin

	Nitrogen content	Heat-coagulable nitrogen	Carbohydrate content*	Sedimentation constant × 10^{13}	Mobility × 10^5 at pH 7.35 ionic strength 0.1
	per cent	*per cent*	*per cent*		
Antitoxic pseudoglobulin†	14.34	81.9	2.55	7.2	2.6
Inactive pseudoglobulin	14.32	81.3	2.58	7.2	2.6
Specific precipitate	14.55	—	2.48	—	—

* By method of Sørensen and Haugaard (28). Calculated as galactose-mannose-glucosamine.

† 43.5 per cent of nitrogen specifically precipitable by toxin.

In Fig. 2 are shown scale displacement-distance sedimentation diagrams for α-globulin-free antitoxic pseudoglobulin before and after absorption with pure diphtheria toxin.

The original α-globulin-free antitoxic pseudoglobulin, the absorbed pseudoglobulin, prepared as above, and the washed floccules dissolved in dilute acetic acid were then thoroughly dialyzed against distilled water and analyzed for nitrogen content, carbohydrate content, and heat-coagulable nitrogen. Heat coagulation was carried out in dilute solution containing

TABLE III

Summary of Ultracentrifuge Experiments on Mixtures of Toxin and Antitoxic Pseudoglobulin in Inhibition Zones

Experiment No.	Toxin	Toxin nitrogen	Anti-toxin	Total pseudo-do-glob-ulin	Sedimentation constant × 10^13			Areas under boundaries*			Area combined toxin	Area combined anti-toxin
					s_1	s_2	s_3	A_1	A_2	A_3		
	Lf per cc.	mg. per cc.	units per cc.	N per cc.				sq. in.	sq. in.	sq. in.		
35	0	0	275	1.135	—	6.92	—	—	41.8	—	—	—
46	0	0	275	1.135	—	7.10	—	—	38.6	—	—	—
36	0	0	0	0.637	—	7.04	—	—	16.1	—	—	24.1
38	95	0.045	275	1.135	—	7.57	—	—	18.3	—	—	21.9
39	95	0.045	0	0.637	—	7.29	—	—	16.2	—	—	24.0
43	48	0.023	275	1.135	—	7.23	(ca. 25)	—	23.8	—	—	16.4
43 b†	48	0.023	275	1.135	—		(ca. 25)					
41	1160	0.533	275	1.135	4.45	7.35	9.34	2.2	18.3	ca. 2.0	12.5	21.9
42	1160	0.533	0	0.637	4.71	7.03	—	14.7	16.8	—	0	23.4
45	2100	0.965	275	1.135	4.47	7.15	8.91	17.6	20.0	16.1	8.7	20.2
44	2100	0.965	0	0.637	4.09	6.70	—	26.3	17.3	—	0	22.9
6‡	1500	0.69	300	0.50	4.49	—	9.92					
8‡	1500	0.69	300	0.50	5.07	—	10.54					
10‡	1500	0.69	300	0.50	4.90	—	10.63					
13‡	1420	0.65	237	0.40	5.03	—	9.41	8.4				
20§		ca. 0.035		1.19	—	8.0	—					

The areas (A_1, A_2, A_3) are calculated from the measured areas under the sedimentation curves according to McFarlane (30). They have not been corrected for the effect of changing optical scale distance (see Lamm, O., *Nova Acta Regiae Soc. Scient. Upsaliensis*, 1937, 15). The apparent discrepancy between the percentage antitoxin in our pseudoglobulin preparation found by nitrogen determination and that found by analysis of the ultracentrifuge data may be attributed to our failure to apply this correction. However, the magnitude of the possible error is not sufficient to affect the conclusions drawn from the above figures.

s_1 = toxin, s_2 = antitoxic or inactive pseudoglobulin, s_3 = reaction product.

* Inactive toxin absorbed pseudoglobulin areas and nitrogen contents corrected for small dilution factor.

† Run at 20,000 R.P.M.; all other runs at 50,000 and 60,000 R.P.M.

‡ Specific floccules dissolved in excess pure toxin.

§ Floccules dissolved in excess antitoxic pseudoglobulin.

0.7 per cent salt and 15 per cent alcohol. Table II compares various properties of the antitoxic pseudoglobulin before and after specific absorption with toxin. No significant differences of any kind were detected.

Mixtures of Toxin and Antitoxin for Study in Ultracentrifuge.—Both toxin and antitoxin were dialyzed against the buffer containing 1 per cent salt described in the preceding paragraph. In most experiments, the final antitoxin titer was held constant

at 275 units per cc. 1 cc. of toxin of the desired Lf or nitrogen content was added rapidly with mixing to 1 cc. of antitoxic pseudoglobulin (550 units per cc.) and allowed to remain in the cold for at least 24 hours prior to being sedimented. Control mixtures were made by using the inactive toxin-absorbed pseudoglobulin described in the foregoing paragraph. On the other hand, the washed specific floccules dissolved in excess toxin or antitoxin were allowed to stand at least 5 days at room temperature and then in the cold in order that resolution might be complete.

In Table III is given a summary of the results of the more important ultracentrifuge experiments with mixtures of toxin and antitoxic pseudoglobulin in the soluble inhibition zones of toxin and antitoxin excess. In Figs. 3 and 4 are shown two typical scale displacement-distance diagrams in regions of slight and large toxin excess.

DISCUSSION

Preparations of diphtheria antitoxic horse pseudoglobulin have been made of which 43.5 per cent of the nitrogen is specifically precipitable by toxin. These preparations behave in every way, except immunologically, as a single molecular species; they are extremely homogeneous by sedimentation, electrophoresis and diffusion. Although the molecular weight of antitoxin, as calculated from the sedimentation-diffusion data, does not differ significantly from that of the normal horse serum globulins (von Mutzenbecher, 29; McFarlane, 30; Svedberg, 31), the electrophoretic mobility of antitoxic pseudoglobulin does differ from that of the normal serum components. It seems probable that a pseudoglobulin with similar mobility is present in normal serum in very low concentration and that this fraction is enormously increased on immunization. In preliminary experiments using diluted whole antitoxic serums, we have found this new component to be the predominating one while the β-component of Tiselius has practically disappeared. The diffusion curves obtained by plotting the scale displacements were analyzed by the method of moments and the points were found to lie on an ideal distribution curve within the limits of measurement of the displacement. So far as we are aware, this is the first time that ideal diffusion has been obtained with a serum globulin prepared by fractionating whole serum. Thus although two substances are known to be present in nearly equal amount, *i.e.* diphtheria antitoxin and inactive pseudoglobulin, they cannot be distinguished from one another by size, shape, or charge. The sedimentation constant and mobility of the inactive pseudoglobulin remaining after absorption of antitoxin with pure toxin remains identical with the material before absorption. Finally, the carbohydrate and nitrogen contents of the original preparation, of the

toxin-absorbed inactive pseudoglobulin, and of the specific floccules are the same after correcting the last for their toxin content.   Incidentally, our carbohydrate determinations on specific floccules are in good agreement with those reported by Hewitt (32).   In view of all these findings, the isolation of immunologically pure antitoxin from serum by chemical fractionation would appear a remote if not impossible accomplishment.   The properties which distinguish antitoxin from associated inactive pseudoglobulin must lie in the intimate steric configuration of the surface of the molecules.

Since both toxin and antitoxin have now been obtained as homogeneous proteins of known molecular weight, it becomes possible to calculate the average molecular composition of the floccules precipitated in the equivalence zone.   Furthermore, from a study of the areas under sedimentation diagrams of mixtures of toxin and antitoxin in the soluble inhibition zones, the maximum "valence" of toxin and antitoxin with respect to each other may be calculated.   The ratio of antitoxin nitrogen to toxin nitrogen in the precipitate formed at the flocculation point is 3.6:1.[4]   Since the nitrogen factor for antitoxin is slightly higher than that for toxin, 6.95 as compared with 6.25, the actual ratio of antitoxin to toxin at the flocculation point is 4.1:1.   From the molecular weights of toxin and antitoxin, *i.e.* 70,000 and 150,000 respectively, the ratio of antitoxin to toxin in a complex of composition TA_2 is readily calculated to be 4.3:1.   It seems reasonable, therefore, to consider $(TA_2)_x$ as the average molecular formula for the specific precipitate at the flocculation point and to calculate the composition of the floccules at other reference points on this basis.   This has been done in Table IV and the composition compared with the horse serum albumin-anti-serum albumin system in the rabbit (Heidelberger, 7) where the molecular weights of the two reactants are nearly the same as toxin and antitoxin.   The analogies and differences between the flocculation reaction and a typical precipitin reaction are apparent from this table. The formation of soluble complexes at both ends of the equivalence zone in the toxin-antitoxin system has already been emphasized.   It is also evident that although the region of precipitation is far wider in the precipitin reaction, the actual equivalence zone or region of complete neutralization has narrowed considerably.   The actual composition of the complexes formed between antigen and antibody are closely analogous in the two systems.

[4] We previously assigned the value 3.5 for this ratio, but subsequent work on a number of antitoxic preparations inclines us to favor a slightly higher value.

From a study of Table III it will be noted that the area of combined antitoxin, which is proportional to the concentration of bound antitoxin, is the same for 275 units regardless of whether 95 Lf or 2100 Lf of toxin (0.045 to 0.965 mg. toxin nitrogen) has been added. The average area is 23 sq. in. In terms of molecules, this is equivalent to ratios varying from one molecule of toxin to 6 of antitoxin up to 7.6 of toxin to one of antitoxin. In Experiment 43, however, only 48 Lf (0.023 mg. nitrogen) of toxin were present per 275 units of antitoxin and the area of combined antitoxin was only 16.4 sq. in., or about two-thirds the total antitoxin. Since in this case the molecular ratio of antitoxin to toxin was 12:1, the maximum number of molecules of antitoxin that can combine with one of toxin would appear to be 8. This may be termed the maximum valence of toxin for antitoxin. Similar examination of experiments in the toxin inhibition zone (toxin ex-

TABLE IV

Comparison between Molecular Composition of Toxin-Antitoxin Complexes with Serum Albumin-Anti-Serum Albumin System (Heidelberger, 7)

Region of large antibody excess*	Antibody and equivalence zone	Flocculation point	Antigen and equivalence zone	Large antigen excess
(TA$_8$)	TA$_4$	TA$_2$	T$_2$A$_3$	(TA) and (T$_2$A)
SaA$_6$	SaA$_4$	—	SaA$_3$	(SaA)

The serum albumin system was studied in the rabbit.

* Formulas in parentheses indicate soluble complexes in inhibition zone.

cess) show that the maximum valence of antitoxin for toxin is definitely greater than one and at most two. In Fig. 3 and Experiment 41 of Table III, a small peak with $s = 9.54 \times 10^{-13}$ is present in addition to those corresponding to excess free toxin and inactive pseudoglobulin. The new peak apparently corresponds to either TA or T$_2$A. When a still greater excess of toxin is present, the area under the new peak is markedly increased, as shown by Experiment 45, Table III, and illustrated in Fig. 4. Even in this case, however, the area under the new component does not account for all the toxin and antitoxin used up in the reaction. The remainder has formed large aggregates which sediment to the bottom of the cell too rapidly to be observed. Thus the closer we approach the equivalence zone, the higher the proportion of toxin-antitoxin complex present in the aggregated form, until finally when the neutral zone is reached, the aggregates become insoluble. An analogous situation exists in the antitoxin inhibition zone. In this case, the complex TA$_8$ could be detected in the presence of a large excess of antitoxin, by running the centrifuge at low speed (Experiment

43 *b*). The boundaries were quite polydisperse and resolution was not satisfactory enough to enable us to calculate an accurate sedimentation constant. It is clear, however, that the complex formed is large, with a molecular weight in the millions. So far as our experiments go, they suggest that the equilibrium between aggregated and unaggregated forms of the toxin-antitoxin complex as well as its composition, is dependent on the relative proportions of the two reactants added. It may also be noted that similar results are obtained using washed specific floccules dissolved in

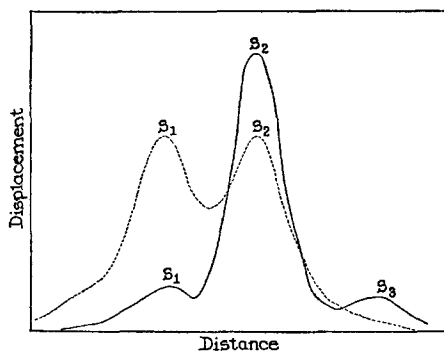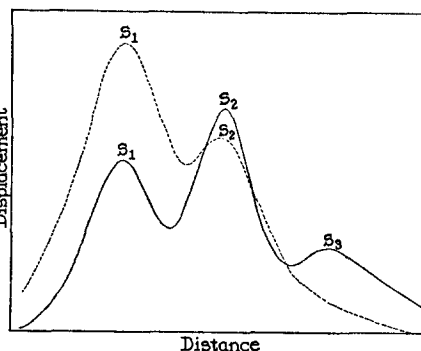FIG. 3   FIG. 4

FIG. 3. Sedimentation diagram of mixture of toxin and antitoxic pseudoglobulin in region of toxin excess. Heavy line: 0.533 mg. toxin nitrogen per cc. (1160 Lf per cc.) and 1.135 mg. pseudoglobulin nitrogen per cc. (275 units per cc.) of which 0.637 mg. is inactive pseudoglobulin. Broken line: 0.533 mg. toxin nitrogen per cc. and 0.500 mg. inactive toxin-absorbed pseudoglobulin nitrogen per cc.

FIG. 4. Sedimentation diagram of mixture of toxin and antitoxic pseudoglobulin in region of toxin excess. Heavy line: 0.965 mg. toxin nitrogen (2100 Lf per cc.) and 1.135 mg. pseudoglobulin nitrogen per cc. (275 units per cc.) of which 0.637 mg. is inactive pseudoglobulin. Broken line: 0.965 mg. toxin nitrogen per cc. and 0.500 mg. inactive toxin-absorbed pseudoglobulin nitrogen per cc.

excess toxin. Since our results show in addition that both toxin and antitoxin are multivalent with respect to one another, it seems probable to us that the aggregation is due to a specific reaction in conformity with the lattice theory of immune reactions (Marrack, 1; Heidelberger, 7).

In the previous study of flocculation, it was shown that the Danysz effect could be explained by assuming that toxin and antitoxin first combine rapidly and reversibly to form a soluble intermediate compound, followed by a slow reaction resulting in flocculation when the proportions are suitable. It seems probable from the results of the present study that the slow reaction postulated is due to aggregation of these intermediates, since the

soluble complexes formed in the regions of toxin and antitoxin excess, become larger as the proportions approach more nearly those of the equivalence zone. The earlier experiments on the Danysz effect, in the light of the present results, demonstrated only the existence of complexes of composition TA_4 in the antitoxin inhibition zone. Therefore, the complexes containing more antitoxin per molecule of toxin, which are now known to exist, must readily combine with further toxin until the more stable composition $(TA_4)_n$ is reached.

In the introduction to this paper it was pointed out that the exceptional character of the flocculation reaction lies chiefly in the soluble inhibition zone in the region of slight antitoxin excess. A possible explanation of this peculiarity has occurred to us based on the following evidence. In the previous work on the quantitative toxin-antitoxin reaction (11) it was shown that antitoxin which had been partially digested with pepsin at an unfavorable pH by the Parfentjev (33) process, contained nearly twice as many units per milligram of specific antibody nitrogen as undigested material. It would appear that the antitoxic groups are distributed in an unsymmetrical manner on the antitoxin molecule and that a large inactive portion of the molecule is split off by treatment with the enzyme. This work was independently confirmed and extended by Pope and his collaborators (34). Further evidence that the antitoxic groups are asymmetrically situated and lie fairly close together can be inferred from the work of Porter and Pappenheimer (35) on the toxin-antitoxin reaction on adsorbed monolayers. There it was found that antitoxin could react specifically with a monolayer of adsorbed toxin but that in the reverse experiment toxin failed to react with a monolayer of antitoxic pseudoglobulin. In the case of other precipitating systems such as pneumococcus polysaccharide-antipolysaccharide, specific reaction occurs regardless of the order of application. Finally it is of interest to note that Lundgren (36) has reported changes in sedimentation behavior of antitoxic pseudoglobulin in solutions of low electrolyte content and has suggested that an equilibrium may exist between molecules of different sedimentation constants. From the foregoing evidence it appears likely that the antitoxic groups may lie fairly close together on one side only of the antitoxin molecule, in contrast to other precipitating antibodies, and therefore the formation of a lattice leading to precipitation is impossible, from steric considerations, in regions of antitoxin excess where toxin molecules are saturated with antitoxin.

SUMMARY

Purified diphtheria antitoxic horse pseudoglobulin has been prepared which is homogeneous by sedimentation, diffusion, and electrophoresis.

Immunologically, however, the preparation contains only 43.5 per cent antitoxin specifically precipitable by toxin. The inactive pseudoglobulin remaining after specific precipitation was found to have the same physical and chemical properties as the original antitoxic pseudoglobulin. Although the molecular weight of antitoxin is the same as that of the normal horse serum globulins, the electrophoretic mobility does differ from those normally present.

The molecular weight of diphtheria toxin is 70,000 and of antitoxin is 150,000.

From ultracentrifuge studies on the two reactants and on mixtures of toxin and antitoxin in the soluble inhibition zones, the average molecular composition of the specific floccules at certain reference points throughout the equivalence zone and the maximum "valence" of toxin and antitoxin with respect to each other have been calculated.

The significance of the results has been discussed in relation to antigen-antibody reactions in general and a possible explanation for the exceptional behavior of the toxin-antitoxin reaction in the region of excess antitoxin has been suggested.

BIBLIOGRAPHY

1. Marrack, J. R., Chemistry of antigens and antibodies, *Great Britain Med. Research Council, Special Rep. Series, No. 230*, 2nd edition, 1938.
2. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62**, 697.
3. Heidelberger, M., *Bact. Rev.*, 1939, **3**, 49.
4. Heidelberger, M., Kendall, F. E., and Teorell, T., *J. Exp. Med.*, 1936, **63**, 819.
5. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1936, **64**, 161.
6. Heidelberger, M., and Pederson, K. O., *J. Exp. Med.*, 1937, **65**, 393.
7. Heidelberger, M., *J. Am. Chem. Soc.*, 1938, **60**, 242.
8. Kabat, E. A., *J. Exp. Med.*, 1939, **69**, 103.
9. Tiselius, A., *Biochem. J.*, 1937, **31**, 1464.
10. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, 1939, **69**, 119.
11. Pappenheimer, A. M., Jr., and Robinson, E. S., *J. Immunol.*, 1937, **32**, 291.
12. Eaton, M. D., *J. Bact.*, 1936, **31**, 367.
13. Pappenheimer, A. M., Jr., *J. Biol. Chem.*, 1937, **120**, 543; 1938, **125**, 201.
14. Danysz, J., *Ann. Inst. Pasteur*, 1901, **16**, 331.
15. Healey, M., and Pinfield, S., *Brit. J. Exp. Path.*, 1935, **16**, 535.
16. Eagle, H., *J. Immunol.*, 1937, **32**, 119.
17. Pope, C. G., and Healey, M., *Brit. J. Exp. Path.*, 1938, **19**, 397.
18. Lundgren, H. P., Pappenheimer, A. M., Jr., and Williams, J. W., *J. Am. Chem. Soc.*, 1939, **61**, 533.
19. Chow, B. F., and Goebel, W., *J. Exp. Med.*, 1935, **62**, 179.
20. Green, A. A., McKhann, C. E., Kapnick, I., and Fahey, K., *J. Immunol.*, 1939, **36**, 245.
21. Kendall, F. E., *J. Clin. Inv.*, 1937, **16**, 921.
22. Ramon, G., *Compt. rend. Soc. biol.*, 1923, **88**, 167.

23. Locke, A., and Main, E. R., *J. Infect. Dis.*, 1926, **39**, 484.
24. Glenny, A., and Pope, C. G., *J. Path. and Bact.*, 1927, **30**, 587.
25. Modern, F., and Wernicke, R., *Compt. rend. Soc. biol.*, 1930, **105**, 119.
26. Pope, C. G., and Healey, M., *Brit. J. Exp. Path.*, 1939, **20**, 213.
27. Lamm, O., and Polsen, A., *Biochem. J.*, 1936, **30**, 528.
28. Sørensen, M., and Haugaard, *Biochem. Z.*, 1933, **260**, 247.
29. von Mutzenbecher, P., *Biochem. Z.*, 1933, **266**, 226, 256.
30. McFarlane, A. S., *Biochem. J.*, 1935, **29**, 407.
31. Svedberg, T., *Ind. and Eng. Chem., Analytical Edition*, 1938, **10**, 113.
32. Hewitt, L. F., *Biochem. J.*, 1934, **28**, 2080.
33. Parfentjev, I. A., United States patent 2065196, 1936.
34. Pope, C. G., *Brit. J. Exp. Path.*, 1938, **19**, 245.
35. Porter, E. F., and Pappenheimer, A. M., Jr., *J. Exp. Med.*, 1939, **69**, 755.
36. Lundgren, H. P., *Nature*, 1939, **143**, 896.