STUDIES ON DIPHTHERIA TOXIN AND ITS REACTION WITH ANTITOXIN[1]

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It has been customary for the recipient of this award to give a resume of his own work.[2] I hope that it will not be considered inappropriate if I preface this paper with a few remarks of a more general nature.

Since the discovery of diphtheria toxin by Roux and Yersin in 1888 and the discovery of antitoxin two years later by von Behring and Kitasato a vast amount of literature has accumulated and the diphtheria problem may well be considered as a classical problem in bacteriology and immunity. Thus, diphtheria toxin has become a model to which the nature and behavior of other bacterial toxins have been related and until about 1925, the diphtheria toxin-antitoxin reaction served as a prototype to which other antigen-antibody systems were compared. However, during the decade which followed the introduction of formol toxoid and the flocculation reaction by Georges Ramon, relatively little progress was made and I think that a notable shift of interest away from the diphtheria problem became evident during that period. It is interesting to examine some of the reasons for this change of attitude. Probably there were many who regarded the diphtheria problem as solved because the disease was no longer a leading

[1] Read before the Forty-second Meeting of the Society of American Bacteriologists at Baltimore, Maryland, following the presentation of the Eli Lilly and Company Research Award in Bacteriology and Immunology for 1941.

[2] Needless to say, all of the work under discussion was carried out with the help and collaboration of others. Of the many concerned I would particularly like to mention Dr. Elliott S. Robinson to whom I am indebted for his continued interest and many valuable suggestions while I was working at the Antitoxin and Vaccine Laboratory, Jamaica Plain, Massachusetts, and Prof. J. W. Williams in whose hospitable laboratory at the University of Wisconsin the physicochemical studies were carried out.

cause of death. In my opinion, however, the chief cause for the shift of interest after 1925 was that the diphtheria problem had reached a point where it became imperative to isolate diphtheria toxin in pure form before further steps could be taken. Until pure toxin became available, little further information as to its nature or the reasons for its toxicity was to be expected. Moreover, it was difficult to conceive of much further progress towards an understanding of the mechanism of the toxin-antitoxin reaction until at least one of the reactants was available in pure form. Finally, the isolation of the stable pneumococcus polysaccharides, which could be easily purified and chemically characterized and which were protein-free, caused the polysaccharide-antipolysaccharide system to supplant the toxin-antitoxin reaction as the model antigen-antibody system.

The fundamental work of Mueller and his coworkers on the nutrition of the diphtheria bacillus opened up a new approach to the isolation and characterization of diphtheria toxin; and the development of *absolute* quantitative methods for study of the precipitin reaction by Heidelberger and Kendall offered a new method for the analysis of the mechanism of the toxin-antitoxin reaction. Moreover, since 1925 many new methods for handling and characterizing small quantities of proteins have been developed. It is interesting and not surprising, therefore, that in 1936–1937 two independent workers in this country, Eaton (1936) and ourselves (1937a), reported the isolation of diphtheria toxin and two other laboratories abroad described independently the isolation of diphtheria toxoid (Boivin and Izard, 1937; Theorell and Norlin, 1937). All the preparations were roughly of the same activity and degree of purity although prepared on different kinds of media and by different fractionation procedures.

So long as culture media consisted of peptones and meat extractives the separation and isolation of small amounts of biologically active proteins, such as diphtheria toxin, in pure form and in sufficient quantity to characterize, was a problem presenting almost hopeless difficulties. Mueller's medium of chemically defined composition (1940) containing no substance with molecular weight higher than the simple amino acids, offered a means

of avoiding many of the difficulties. Our first experiments, which were carried out at the Massachusetts Antitoxin and Vaccine Laboratory, were, therefore, concerned with the production of diphtheria toxin on Mueller's medium. Although these experiments at first met with complete failure, it was eventually found that the iron concentration is of great importance in determining the yield of toxin (Pappenheimer and Johnson, 1936). Although traces of this element are essential for growth of the diphtheria bacillus, slightly higher concentrations are sufficient to cause almost complete inhibition of toxin production. The toxin production rises steeply to an optimum at about 0.08 mg. iron per liter, and then falls almost as rapidly, so that when 0.5 mg. iron is present per liter, only traces of toxin are produced. The early failure to produce toxin on Mueller's medium was due to an excess of iron present as impurity in the substances used to make up the medium. The formation of a calcium phosphate precipitate at pH 7.6–7.8 in the medium resulted in removal of this inhibitory iron and potent toxin was produced. These observations on the effect of iron on toxin production were not new and had previously been noted by others who were apparently not fully aware of their importance. In fact we agree with Mueller (1941b) that the careful control of the iron concentration is undoubtedly the most important single factor concerned with toxin production on any sort of medium which will support normal growth of the diphtheria bacillus. We have been careful not to state that excess iron causes *complete* inhibition of toxin production, because Mueller has recently shown that small amounts of toxin are produced at concentrations of iron equivalent to those found in tissues. Under these conditions he has made the important observation that *gravis* strains produce considerably more toxin than *mitis* strains of *Corynebacterium diphtheriae*. At low iron concentrations the reverse is true (Mueller, 1941c).

The production of potent diphtheria toxin on a chemically defined medium dispelled the notion, currently held by many, that peptone or partial protein cleavage products were essential for toxin production and provided an excellent source for isolation of the toxin. Several hundred liters of diphtheria toxin were there-

fore produced on a medium consisting of a complete acid hydrolysate of purified gelatin, supplemented with the amino acids methionine, cystine and tryptophane, with lactic acid, glucose and maltose, inorganic salts and the accessory growth factors nicotinic acid, pimelic acid and beta-alanine. The average yield of toxin on this medium was 40–50 Lf/ml. or about 2500 m.l.d. per ml.[3] The only proteins present in the culture filtrate were those elaborated by the diphtheria bacillus itself and it therefore proved possible to prepare a highly purified toxin by concentration *in vacuo*, neutral salt fractionation, adsorption of certain impurities with alumina cream and by dialysis. The procedure was a lengthy and purely empirical one and I will not weary you with its details. The isolation of the toxin was facilitated because there were available accurate animal and serological methods for testing. Thus, the Ramon flocculation reaction can be used to determine the amount of toxin present in a given sample to within 3–5 per cent. The greatest difficulty has been in obtaining sufficient purified material with which to work and in proving that the final product is free from other bacterial proteins and is in fact identical with the actual toxin. Altogether we have isolated about 25 grams of a toxic protein which we believe to be 95–98 per cent pure diphtheria toxin for reasons which will be discussed presently.

Some of the analytical data on purified diphtheria toxin protein as isolated by Eaton and by ourselves are given in table 1. In spite of the great differences in the culture medium used (Eaton's contained 2 per cent Proteose peptone) and in the methods of fractionation, the agreement is fairly satisfactory. It is clear from this table, that there is little to distinguish diphtheria toxin from other proteins from whatever source and that only by analysis of a large series of properties can it be characterized so as to differentiate it from other proteins. Moreover, there is nothing

[3] Recently Mueller (1941a) has improved the medium for toxin production still further. Using his new formula, 100 Lf/ml. or 5000 m.l.d. per ml. of toxin may be routinely produced. Since 1937 all of the toxoid for human immunization prepared at the Massachusetts Antitoxin and Vaccine Laboratory has been made from toxin produced on a medium of chemically defined composition.

in the data summarized which gives any clue as to why the protein is so extremely toxic. Parenthetically, it may be noted that all of the amino acids found in the isolated protein were synthesized by the strain used and are not essential for its growth.

The purified toxic protein from the diphtheria bacillus is extremely sensitive and rapidly undergoes denaturation. Even in the cold the toxin is irreversibly damaged in solutions more acid

TABLE 1

Biological activity and chemical composition of purified diphtheria toxin

CONSTITUENTS	M.D.E.	A.M.P.
	per cent	*per cent*
M.L.D. per mg	10,000	14,000
Nitrogen per Lf	0.00046–0.00055	0.00046
Carbon		51.47
Hydrogen		6.75
Nitrogen*	16	16.0
Sulfur	?	0.75
Phosphorus	<0.5	<0.05
Ash		1.4
Amino nitrogen	2–2.5	0.98
Tyrosine	+†	9.5
Tryptophane	+†	1.4
Arginine	+†	3.8
Histidine		2.4
Lysine		5.3
Specific rotation	−45°	−39°
pH of isoelectric point	Between 4 and 5‡	4.1§
Molecular weight		72,000

* Corrected for ash content.

† Color test.

‡ Minimum solubility.

§ Cataphoresis.

than pH 5.6 and in the neutral region is destroyed by short exposure to 56°C. or even by shaking in dilute unbuffered solution at room temperature. It has been shown by Eaton that the flocculation time, that is the time of formation of the first flocculating mixture of diphtheria toxin and antitoxin of known strength under controlled conditions, is an extremely sensitive index as to whether any denaturation has taken place and this has proved a

convenient and useful guide during fractionation. Denaturation is manifested by loss of toxicity, increased flocculation time and decreased solubility.

We have mentioned that no prosthetic grouping has been discovered, as yet, which can in any way account for the toxicity of diphtheria toxin. In this respect the toxic protein resembles other active proteins such as insulin and the proteolytic enzymes. Numerous agents have been found which destroy its toxicity

TABLE 2

Acetylation of diphtheria toxin

(Reprinted from J. Biol. Chem., 1938, **125**, 204)

TIME OF ACETYLATION	AMINO N			TYROSINE + TRYPTOPHANE		M.L.D. PER MG.	Kf_{30}*
	Van Slyke	Acetylated	Formol titration	pH 11 method	pH 8 method		
min.	*per cent total N*	*per cent total N*	*per cent total N*	*per cent*	*per cent*		
0	6.1†	0	6.6	10.5	10.5	14,000	15
4	2.6	3.5‡	3.2	10.1	9.4	40	75
10	3.0	3.1‡	3.1	10.4	8.3	7	200
40	2.6	3.5	3.0	10.1	6.6	7	<600§

* Kf is taken to represent the flocculation time in minutes at 42°. The subscript denotes the flocculation titer in Lf units per ml.

† This figure is the average of five determinations.

‡ The reduction in amino nitrogen as calculated from the lysine content is 3.2 per cent.

§ There was no flocculation after 40 hours in the cold and 10 hours at 42°, but the solution was slightly cloudy in the expected zone. After it was blended with a rapidly flocculating toxin, about 30 per cent flocculated in 80 minutes, indicating a 70 per cent loss in combining power.

without seriously injuring its immunological properties. These include iodine, formaldehyde, certain diazo compounds (Pirosky and Friedheim, 1941) and ketene (Goldie, 1937). In particular, the action of ketene and dilute formaldehyde on the highly purified toxin has been studied. The ketene experiments are summarized in table 2 which shows that the loss of toxicity is associated with a decrease in the amino nitrogen content closely approximating that calculated from the lysine content. Further treatment with ketene results in acetylation of the tyrosine

hydroxyl groups of the protein as shown for pepsin by Herriot (1935). Almost all of the immunological activity is lost when the apparent tyrosine content has been reduced by 35 per cent. While it cannot be claimed that the ϵ-amino groups of lysine are toxic in themselves, it is suggested that they are concerned with toxicity by virtue of their spatial arrangement in the molecule as a whole. Similar experiments with formaldehyde as detoxifying agent, suggest that the mechanism is analogous to that of ketene.

Evidence that the protein which has just been described is identical with the actual toxin itself rests on the following facts:

1. It has not been possible to alter a large series of analytical and biological properties by further fractionation of the purified material.

2. In so far as comparison is possible, the properties of the toxic protein (or toxoid) prepared by different investigators using totally different types of media and fractionation procedures agree closely.

3. In culture filtrates containing enough iron to inhibit toxin production almost completely, no appreciable protein comes down with ammonium sulfate in the fraction giving a precipitate when toxin is present.

4. Any loss in activity is invariably accompanied by a corresponding increase in material of lower solubility.

5. The toxic protein shows a single homogeneous boundary by sedimentation in the ultracentrifuge, $s_{20} = 4.6$ Svedberg units, and shows ideal diffusion, $D_{20} = 6.0 \times 10^{-7}$ cm.^{2}/sec. It also shows a single homogeneous boundary in the Tiselius electrophoresis apparatus and it has been demonstrated that the toxicity moves with the observed boundary.

6. The remaining evidence rests on immunological data. In the first place it can be shown that the toxic protein is completely precipitated by antitoxin. Still more conclusive evidence may be derived from a quantitative study of the flocculation reaction between toxin and antitoxin (Pappenheimer and Robinson, 1937b). If we plot milligrams of nitrogen flocculated, against Lf units of toxin added to a constant amount of antitoxin, a straight line curve is obtained throughout most of the flocculation or

equivalence zone (curve A, fig. 1). The slope of this straight line is equal to the toxin nitrogen per Lf or flocculating unit which in turn has the same value as that reached by actual isolation of the toxin. The identical slope and amount of precipitate is obtained regardless of the purity of either the toxin or antitoxin preparations used. Because of this close agreement between the im-

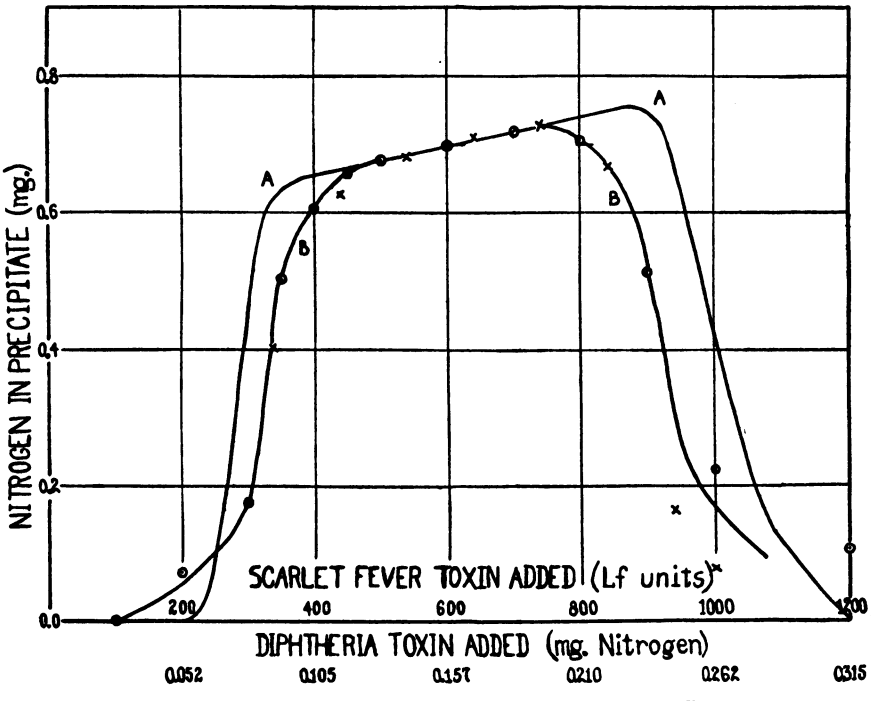

FIG. 1. QUANTITATIVE TOXIN-ANTITOXIN FLOCCULATION REACTION

Curve A: Diphtheria system. Curve B: Scarlet fever system. (Reprinted from J. Exptl. Med., 1941, **74**, 550.)

munological and chemical results, it seems most improbable that the toxicity of the protein isolated from the bacterial culture filtrates can be due to mere traces of a more highly toxic substance present as an impurity. This is always a real possibility in work of this nature and one which is often difficult to rule out by other means.

Quantitative studies of the flocculation reaction may be used

to estimate the purity of other biologically active protein preparations. With G. A. Hottle (1941), we have recently studied the scarlet fever toxin-antitoxin reaction and from the slope of the straight line portion of curve B in figure 1, it has been calculated that pure scarlet fever toxin contains 0.00023 mg. nitrogen per Lf unit or about 10^8 skin test doses per mg. of nitrogen.

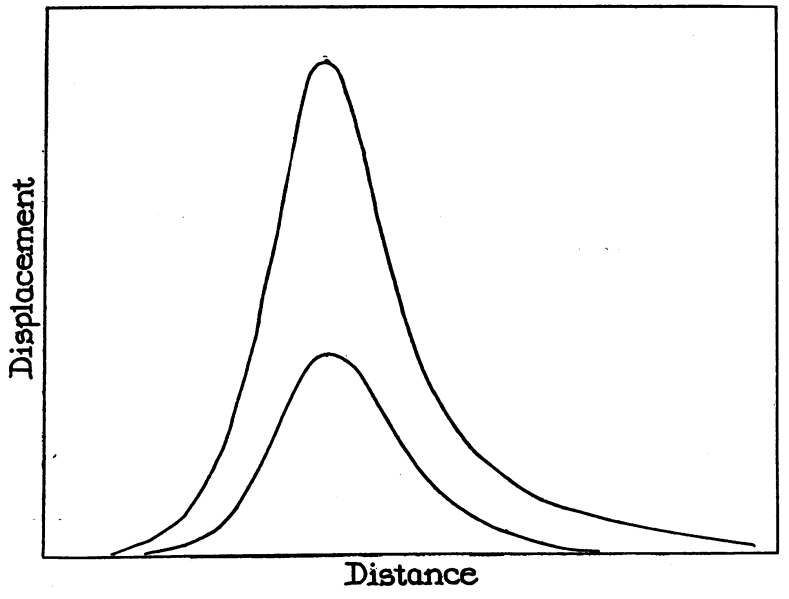

FIG. 2. SEDIMENTATION DIAGRAM OF ANTITOXIC α-GLOBULIN-FREE PSEUDOGLOBULIN BEFORE AND AFTER REMOVAL OF ANTITOXIN BY SPECIFIC PRECIPITATION WITH TOXIN.

(Reprinted from J. Exptl. Med., 1940, 71, 254)

A number of other rather interesting deductions may be made from these curves. The straight line curve obtained through most of the equivalence zone means that precipitation of both toxin and antitoxin must be complete within the zone, a conclusion which is born out by the fact that only traces of toxic or antitoxic activity can be detected in the supernatants using the sensitive rabbit intracutaneous test. Moreover, it must be obvious from the data that toxin and antitoxin combine in several proportions with one another. Since the amount of toxin added is known it

is possible to calculate the proportion of toxin and antitoxin in the specific floccules at various reference points.

It has not been possible to isolate the other component of the system, i.e., antitoxin, in immunologically pure form by fractiona-

TABLE 3

Some properties of antitoxic pseudoglobulin before and after specific absorption with pure diphtheria toxin

(Reprinted from J. Exptl. Med., 1940, **71**, 254)

	NITROGEN CONTENT	HEAT-COAGULABLE NITROGEN	CARBOHYDRATE CONTENT*	SEDIMENTATION CONSTANT $\times 10^{13}$	MOBILITY $\times 10^5$ 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. Calculated as galactose-mannose-glucosamine.

† 43.5 per cent of nitrogen specifically precipitable by toxin.

TABLE 4

Some physical properties of diphtheria toxin and antitoxic pseudoglobulin

(Adapted from J. Physical Chem., 1941, **45**, 1)

PHYSICAL PROPERTIES	TOXIN	ANTITOXIC PSEUDOGLOBULIN	
		Untreated	Dissociated from floccules
Per cent of specifically precipitable nitrogen	95–98	33	77
Specific volume	0.736	(0.745)	(0.745)
Sedimentation constant $\times 10^{13}$	4.6	7.2	5.7
Diffusion constant $\times 10^7$	6.0	3.9	5.0
Molecular weight	71,000	180,000	110,000
Svedberg Dissymmetry No., f/fo	1.22	1.38	1.26
Ratio of major to minor axes	4.7	7.0	5.3
Mobility $\times 10^5$ (pH 7.35; ionic strength 0.1)	4.9	2.6	

tion of antitoxic horse plasma. Nevertheless, a preparation of antitoxic pseudoglobulin has been made which was found homogeneous by sedimentation, electrophoresis and diffusion (Pappenheimer, et al., 1940a). Only 43.5 per cent of this preparation is

specifically precipitable by pure toxin. The behavior of this preparation in the ultracentrifuge is illustrated by the following sedimentation diagrams taken before and after treatment with toxin. Note that no component corresponding to the toxin can be observed; it has been completely precipitated. The *inactive* pseudoglobulin which remains in the supernatant, shows identical chemical and physical properties with the original antitoxic pseudoglobulin and therefore has served as an excellent control for physico-chemical studies on the reaction between toxin and antitoxin. Some of the properties of these preparations are summarized in the tables 3 and 4. These physico-chemical studies have all been carried out in collaboration with Drs. J.

TABLE 5

Comparison between molecular composition of toxin-antitoxin complexes with serum albumin-anti-serum albumin system

(Reprinted from J. Exptl. Med., 1940, **71**, 258)

REGION OF LARGE ANTIBODY EXCESS*	ANTIBODY END EQUIVALENCE ZONE	FLOCCULATION POINT	ANTIGEN END EQUIVALENCE ZONE	LARGE ANTIGEN EXCESS
(TA_8) SaA_6	Ta_4 SaA_4	TA_2	T_2A_3 SaA_3	(TA) and (T_2A) (SaA)

The serum albumin system was studied in the rabbit.

* Formulas in parentheses indicate soluble complexes in inhibition zone.

W. Williams, H. P. Lundgren and Mary Petermann at the University of Wisconsin.

Since the composition of the specific floccules and the molecular weights of the two reacting proteins, toxin and antitoxin, have been determined, we may calculate the average molecular composition of some of their reaction products. In table 5 we have summarized the average molecular composition of toxin-antitoxin complexes at certain reference points as compared with the serum-albumin-antibody system in the rabbit (calculated by Heidelberger, 1938) where the molecular weights of the two reacting proteins are about the same as toxin and antitoxin. From this table, it would appear that the maximum number of toxin molecules that can combine with one of antitoxin is two. Similarly,

the maximum number of antitoxin molecules that can combine with one of toxin is eight. These estimates of what may be termed the maximum "valence" of toxin and of antitoxin, were calculated from the areas under sedimentation diagrams obtained by ultracentrifugation of the reaction products in the soluble inhibition zones of toxin and antitoxin excess. Such studies in

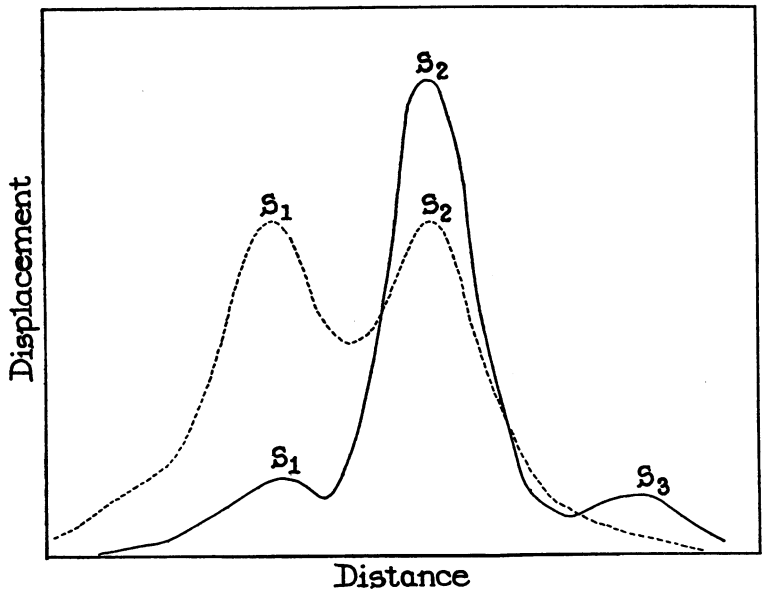

FIG. 3. SEDIMENTATION DIAGRAM OF MIXTURE OF TOXIN AND ANTITOXIC PSEUDOGLOBULIN IN REGION OF TOXIN EXCESS

Heavy line: 0.533 mg. toxin nitrogen per ml. (1160 Lf per ml.) and 1.135 mg. pseudoglobulin nitrogen per ml. (275 units per ml.) of which 0.637 mg. is inactive pseudoglobulin. Broken line: 0.533 mg. toxin nitrogen per ml. and 0.500 mg. inactive toxin-absorbed pseudoglobulin nitrogen per ml. (Reprinted from J. Exptl. Med., 1940, 71, 259.)

the inhibition zones have also demonstrated that considerable aggregation occurs in these regions even though no visible precipitate is formed. Figures 3 and 4 show typical results from sedimentation studies carried out in the region of toxin excess. In addition to free toxin and inactive pseudoglobulin a third component is present which does not appear in the control (dotted

curve). This new and more rapidly sedimenting component evidently represents unaggregated TA or T_2A. The remaining reaction products have aggregated and sediment out too rapidly to be detected at the speed at which these runs were made. The closer we approach to the equivalence zone, the more aggregation occurs until finally when the zone is reached, flocculation takes

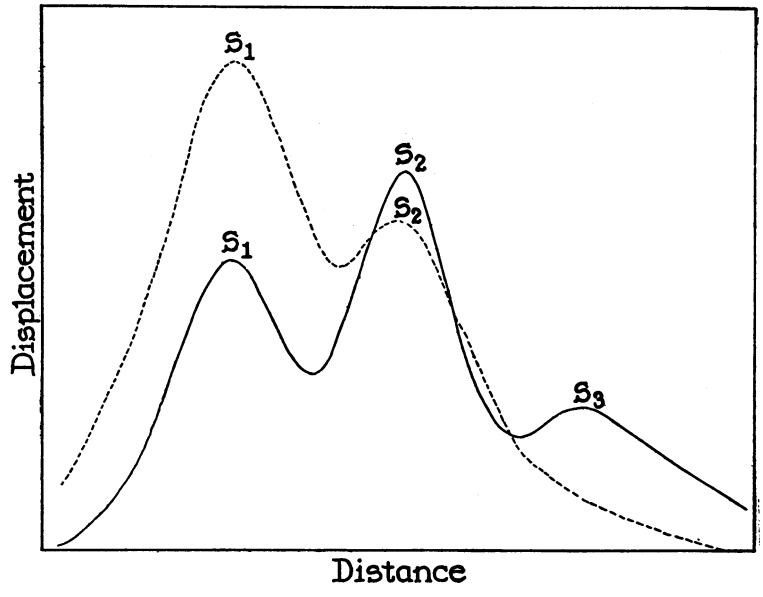

FIG. 4. SEDIMENTATION DIAGRAM OF MIXTURE OF TOXIN AND ANTITOXIC PSEUDOGLOBULIN IN REGION OF TOXIN EXCESS

Heavy line: 0.965 mg. toxin nitrogen (2100 Lf per ml.) and 1.135 mg. pseudoglobulin nitrogen per ml. (275 units per ml.) of which 0.637 mg. is inactive pseudoglobulin. Broken line: 0.965 mg. toxin nitrogen per ml. and 0.500 mg. inactive toxin-absorbed pseudoglobulin nitrogen per ml. (Reprinted from J. Exptl. Med., 1940, 71, 259.)

place. It is interesting that in this region of toxin excess, the same equilibrium between aggregated and unaggregated forms of the toxin-antitoxin complex, may be reached from both sides, i.e., either by mixing toxin and antitoxin directly, or by dissolving the floccules in an excess of pure toxin.

Any theory of the toxin-antitoxin reaction should include an explanation of the following familiar phenomena:

1. The Danysz effect
2. The discrepancy between Ehrlich's L_0 and L_+ doses of toxin
3. The soluble inhibition zone of slight antitoxin excess

In 1901, Danysz observed that less toxin is neutralized by a given amount of antitoxin when the toxin is added in steps rather than all at once. Danysz's original explanation for this phenomenon was simply that toxin and antitoxin may combine in more than one proportion depending upon their relative concentrations. Ehrlich and his followers, however, would not accept Danysz's simple interpretation and proposed an alternative explanation involving the relative affinity of toxin and toxoids for antitoxin The work of Healy and Pinfield (1935) and our own with Robinson (1937b) shows clearly that toxin and antitoxin do combine in many proportions. Moreover, it has been shown that formol toxoid and toxin are quantitatively identical in their reaction with antitoxin. I think, therefore, that the recent work suggests that Danysz's original concept was essentially the correct one. Stated in more modern terms, in light of what we now know about aggregation of the toxin and antitoxin complex, we may write the following equations:

$$\begin{array}{lllll} & & \text{rapid} & \text{slow aggregation} & \\ 1.\ \mathrm{T} + \mathrm{A} & \rightleftharpoons & \mathrm{TA} & \rightleftharpoons & (\mathrm{TA})_w \text{ (soluble)} \\ 2.\ \mathrm{TA} + \mathrm{A} & \rightleftharpoons & \mathrm{TA_2} & \rightleftharpoons & (\mathrm{TA_2})_x \text{ (insoluble)} \\ 3.\ \mathrm{TA_2} + \mathrm{A} & \rightleftharpoons & \mathrm{TA_3} & \rightleftharpoons & (\mathrm{TA_3})_y \text{ (insoluble)} \\ 4.\ \mathrm{TA_3} + \mathrm{A} & \rightleftharpoons & \mathrm{TA_4} & \rightleftharpoons & (\mathrm{TA_4})_z \text{ (soluble in presence of excess A)} \end{array}$$

Note: w, x, y, z represent the average number of molecules in the respective aggregates.

If toxin and antitoxin are added in their proportions at the flocculation point or in those of an L_0 dose, then the first two rapid reactions occur followed by slow aggregation of TA_2 which represents the average molecular composition of the floccules which settle out. However, if only a small amount of toxin is added at first, TA_4 will be the principal product and this will aggregate to a

soluble polymer of high antitoxin content, $(TA_4)_x$. Since the latter aggregation is only slowly reversible, the rest of the toxin, when added, will combine with whatever antitoxin is left to form complexes of low antitoxin content which are in equilibrium with free toxin. Of course, the actual equilibria are undoubtedly much more complex than we have pictured them and the formulas given should be considered as representing an average molecular composition rather than as true chemical formulas. It should also be kept in mind that complexes of intermediate composition such as T_2A_3, T_2A_5, etc., are formed.

The discrepancy between the L_0 and L_+ doses of toxin may be explained in a similar manner. It will be recalled from its definition, that one might expect one L_+ dose of toxin to equal an L_0 dose + 1 m.l.d. Actually, Ehrlich found that 1 L_+ dose = 1 L_0 dose + about 20 m.l.d. and he sought to explain the discrepancy on the basis that toxin is a mixture of compounds of varying degrees of toxicity with different affinities for antitoxin. According to our present ideas, we would explain the apparent discrepancy in the following manner: Complexes of toxin and antitoxin may be formed which contain a higher proportion of toxin than that formed at the exact neutral point. These complexes are somewhat more readily dissociated to yield traces of free toxin (enough to produce skin reactions) than are those at the neutral point. However, an appreciable excess of toxin must be added before there will be sufficient free toxin to cause death of a 250 gram guinea pig in 4–5 days.

A peculiarity of flocculating systems as opposed to precipitating systems is the soluble inhibition zone in the region of slight antibody excess which is characteristic of the former type of reaction. This results in a very narrow zone of actual flocculation and has caused Marrack and others to regard the behavior of the flocculation reaction as altogether abnormal. It occurred to us that the existence of the two types of reaction might be due to a difference in the species used for antibody production. A horse was, therefore, immunized with egg albumin and it was observed that the egg-albumin-anti-egg-albumin system in this animal was analogous in its behavior to the toxin-antitoxin reaction (Pappen-

heimer, 1940b). It seems likely that a narrow zone of flocculation is characteristic of most protein-anti-protein systems in the horse.

It is interesting to speculate on the reasons why no flocculation occurs in the presence of slight antitoxin excess. As we have seen, the antitoxin molecule apparently contains at least two sites or groupings with which toxin molecules may combine. If these reacting sites lie close enough together so that they mutually interact, then it is to be expected that specific aggregation will be limited or impossible in regions of antitoxin excess where toxin molecules are completely covered with molecules of antitoxin. There is a small amount of experimental evidence in favor of this provisional hypothesis. It has been shown by Dr. Petermann (1941) at the Wisconsin Laboratory that when diphtheria antitoxin is treated with pepsin according to Parfentiev's procedure, a new antitoxin is formed with a molecular weight of about half that of the original antitoxin. (See table 4.) The digested antitoxin combines with nearly twice as much toxin per milligram as does untreated antitoxin. These experiments with pepsin suggest that the reacting sites must all be on one half of the original antitoxin molecule since no significant loss in total activity occurs during the digestion. Finally, evidence that the antitoxic groupings are assymetrically situated and lie in fairly close proximity to one another may be inferred from the results of surface film experiments carried out in collaboration with Porter (1939). These experiments have shown that antitoxin will react specifically with an adsorbed monolayer of pure toxin; further application of toxin, however, does not result in the formation of another layer. On the other hand, precipitating systems continue to form multiple alternate monolayers of antigen and antibody.

Of the many problems which still confront us in this field, the most interesting question of all still remains unanswered: Why is diphtheria toxin toxic?

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