ON THE SIZE OF THE TOXIC PARTICLE PASSING THE INTESTINAL BARRIER IN BOTULISM*

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The small intestine appears to be the major site of systemic absorption of toxin in botulism. The absorbed toxin is found in the lymph draining the intestines before it appears in the blood stream and is of a large enough particle size as found in lymph not to be dialyzable *in vitro* (1). The present study has had as its aim the more exact delineation of the particle size of the toxin appearing in the lymph after crossing the intestinal barrier. Conceivably, intestinal action might cause the release of a small sized toxic moiety, if such exists, from the toxic protein molecule. Therefore, an effort has been made to determine whether or not any detectable quantity of the toxin appearing in lymph has dimensions smaller than may be attributed to a protein.

Materials and Methods

Toxins.—Crystalline type A botulinum toxin kindly made available by Dr. E. J. Schantz of Fort Detrick, Maryland, and partially purified toxin of local origin were studied. From 1 to 5 $\times$ 10^6 mouse LD_{50} per ml. were prepared as stock solutions. All dilutions were made in 0.05 M phosphate buffer at pH 6.3 containing 0.2 per cent gelatin.

In some experiments crystalline toxin was exposed in the small intestine by tying off a section of the rat intestine just below the stomach and flushing it with saline. One to 2 ml. of crystalline toxin solution containing a total of 2 to 20 $\times$ 10^5 mouse LD_{50} were introduced, and after 2 hours the toxin was recovered by flushing the small intestine with 8 or 9 ml. of saline. This toxin, called digested toxin, was assayed for toxic potency after ultracentrifugation in a partition cell for determination of the sedimentation coefficient.

Toxicity Assay.—Toxicity was titrated in 6 to 9 week old Namru strain mice by intraperitoneal inoculation of 0.1 ml. of appropriate dilutions in the phosphate-gelatin buffer. The LD_{50} was calculated by the method of Reed and Muench (2).

Protein Assay.—Protein was estimated using the phenol reagent (3) with bovine serum albumin as the standard.

Lymph.—Lymph was obtained from 200 to 300 gm. Long-Evans strain rats which had received toxin intraduodenally. Our experience confirms the observation of May and Whaler

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(1) that ingested toxin upon absorption first appears in the lymph. The lymph was collected by cannulation of the cisterna chyli (4). In the same operation a polyethylene tube for introducing the toxin was inserted through a small incision in the large curvature of the stomach and pushed through the pylorus into the duodenum. The pylorus was tied off to prevent backflow into the stomach. The abdominal incision was closed and the rats were allowed to recover from anesthesia in restraining cages.

After complete recovery from anesthesia 10 to 20 ml. of stock toxin solution, totaling 1 to 5 $\times$ 10^7 mouse LD_{50} per rat, were introduced intraduodenally through the polyethylene tubing in 5 ml. portions at 2 hour intervals. The lymph was collected in containers cooled in cracked ice for a 12 to 24 hour period following the intestinal deposition of the toxin. The collected lymph was clarified by a preliminary centrifugation at about 30,000 *g* for 45 minutes which removed most of the fat and cellular elements without demonstrable loss of toxin.

The amount of toxin appearing in the lymph was of a low order being about 1/100,000th part of the LD_{50} instilled into the rat intestine. Thus the concentration of toxin in lymph was too low for study of the sedimentation characteristics of the toxin by optical methods. Instead, S_{20} values were determined from mouse assay of toxicity of centrifuged specimens of lymph as described in the following section on partition cell centrifugation.

Partition Cell Centrifugation.—While both the Yphantis-Waugh and fixed partition cells were used in the Spinco model E ultracentrifuge, the majority of the determinations were made with the latter cells. Toxicity of the contents of both upper and lower compartments and of the original lymph was determined after the centrifugation. The sedimentation coefficient of the toxin was then calculated (5) using the following equation:—

$$S_{20} = -\frac{1}{2\omega^2 t}\left(\frac{\eta}{\eta_{20}}\right)\ln\left[\frac{X_p^2}{X_m^2} + \frac{c_u}{c_0}\left(1 - \frac{X_p^2}{X_m^2}\right)\right] \tag{1}$$

in which t = total effective time of sedimentation, second; ω = angular velocity, radians per second; X_m = distance from axis of rotation to meniscus, cm.; X_p = distance from axis of rotation to partition, cm.; η = viscosity of solvent at temperature of experiment; η_{20} = viscosity of solvent at 20°C.; C_0 = zero time toxicity obtained by titration of solution placed in centrifuge cell, LD_{50} per ml.; C_u = toxicity of solution removed from upper compartment, LD_{50} per ml. A comparable and independent value for zero time toxicity, C_0', based on titrations in mice of the contents of the upper and lower compartments of the sedimentation chamber after centrifugation can be calculated using the relation,

$$C_0' = \frac{C_u V_u + C_b V_b}{V_u + V_b} \tag{2}$$

in which C_u and V_u are respectively the toxicity and the volume of the contents of the upper compartment, and C_b and V_b are the toxicity and the volume of the contents of the lower compartment.

An approximation of the distance toxic material sedimented, ΔX, was obtained from the equation

$$\Delta X = \frac{C_u}{C_0}(X_p - X_m) \tag{3}$$

The clarified lymph was used directly in the partition cells but, in addition, a number of determinations were made on the supernatant solution and on sedimented material obtained by centrifugation of lymph in a Spinco preparative A rotor at 50,740 R.P.M. (187,000 *g*) for 90 minutes.

Density Gradient Separation.—Density gradient tubes were prepared by layering glycerol

solutions of decreasing concentrations, 1 ml. each, into ½ x 2 inch plastic centrifuge tubes. The gradients used were either 5-10-20-30 or 10-20-40-60 per cent glycerol. One ml. of the toxic lymph was placed on top of the glycerol immediately before centrifugation. Glycerol solutions were prepared by dilution with 0.05 M phosphate buffer at pH 6.3 containing 0.2 per cent gelatin. The practicality of employing glycerol for preparing the density gradient solutions was demonstrated by the finding that exposure of toxin for as long as 2 hours at room temperature to concentrations of glycerol as high as 80 per cent did not result in detoxification.

The filled tubes were centrifuged in a Spinco swinging bucket rotor model SW 39 (about 124,000 *g*) for 1 or 4 hours and decelerated slowly. The fractionation was accomplished by inserting an 18 gauge needle into the tube's bottom and controlling the outflow of solution with an electrolytic device (6) attached to the top of the tube. A constant delivery rate was maintained, and collection tubes were changed at regular intervals.

Electrophoresis.—For electrophoretic analysis clarified, but undiluted lymph was dialyzed against 0.1 M phosphate buffer at pH 7.0 for at least 48 hours. The standard 3 x 25 x 80 mm. Tiselius cell was used with an Aminco electrophoresis apparatus. After the electrophoretic separation the contents of both limbs of the center section were fractionated as described by Heckly (7). A 25 per cent glycerol solution was introduced into the bottom section at the rate of 0.2 ml. per minute and the collecting tubes were changed at 2 minute intervals. On the basis of the photographic record made during the fractionation each fraction was identified as to its position in the cell for correlation with the schlieren pattern, and presence of toxicity.

EXPERIMENTAL

Partition Cell Centrifugation.—Tables I and II record the data of most of the experiments using centrifuge partition cells. The results show that the value of the sedimentation coefficient of toxic material in lymph is not a function of either the force or the time of sedimentation employed (Figs. 1 and 2). The fact that the sedimentation coefficient obtained in experiments in which a high percentage of the toxin in lymph was sedimented are in general agreement with the rest of the values (Fig. 2) is particularly significant because equation (1) is valid only as long as the partition remains in the plateau region; that is, the toxin sedimentation boundary has not moved beyond the partition barrier in the cell. Fig. 2 also indicated a certain degree of homogeneity of the toxic material in lymph since the apparent S_{20} of the toxicity did not change as a function of the percentage of toxin sedimented. This finding would not be made if major fractions of toxicity were associated with particles having markedly different sizes.

Another indication that the particle size of the toxin in lymph was fairly uniform is that the sedimentation coefficient of toxin recovered in both the supernatant and pelleted fractions were essentially the same. As shown most clearly in Fig. 2, there were no significant differences between the sedimentation values of the toxin in the lymph as recovered from the rat and toxin in lymph centrifugally separated into the two fractions. Hence it is probable that the particle size of the bulk of the toxin in lymph was homogeneous.

A possibility exists that the particle size of the toxin in the lymph is the result

TABLE I

Sedimentation Coefficients of the Toxin in Lymph Collected from Rats after Intraduodenal Administration of Botulinum Type A Toxin

Run No.	Preparation	Temperature	Time	R.P.M.	S_{20}	
					Based on observed C_0	Based on calculated C_0'
		°C.	sec.			
54	Clarified crude lymph	1.5	4160	47,660		7.0, 7.5
57	" " "	4.0	3450	47,660		5.7, 5.6
67	" " "	1.6	3600	47,660		6.0
70	" " "	33.5	3400	35,600		6.2, 11.9
71	" " "	25.8	7680	47,660		5.2, 4.8
74	" " "	35	3840	35,600	5.1	8.0
78	" " "	24.5	3840	42,040		8.4
82	" " "	35	3840	42,040	5.0, 4.8	7.9, 6.8
86	" " "	2.5	2880	47,660	15.6, 14.5	7.1, 12.8
94	" " "	5.5	3840	52,640	4.0	>8.9
106	" " "	35	3600	42,040	9.5, 9.2	7.6, 9.2
111	" " "	32	3960	47,660	7.0, 7.5	7.0, 7.4
118	" " "	33.5	3840	47,660	>6.8, 6.7	>7.2, 7.5
121	" " "	29.5	960	47,660	<19.4, <19	<10.6, <10.3
122	" " "	30.5	1920	47,660	13.5, <8.9	8.2, <5.8
80	Supernatant	24.5	3840	42,040	8.0	4.2
84	"	35	3840	42,040	7.2	6.7
88	"	2.5	2880	42,040	9.7	22
97	"	1.0	3840	52,640	3.5	1.8
108	"	35	3960	42,040	>7.1	>5.3
113	"	32	3960	47,660	>7.5	>7.9
80	Pellet	24.5	3840	42,040	8.7	7.8
84	"	35	3840	42,040	6.7	8.5
108	"	35	3960	42,040	>4.6	>5.6
113	"	32	3960	47,660	>7.5	>7.2

TABLE II

Sedimentation Coefficient of Crystalline Type A Botulinum Toxin before and after Intraduodenal Digestion

Run No.	Preparation	Temperature	Time	R.P.M.	S_{20}	
					Based on observed C_0	Based on calculated C_0'
		°C.	sec.			
89	Digested toxin	19.5	3840	42,040	16.6, 16.0	18.7, 17.9
126	" "	29.5	3840	42,040	12.8	12.9
90	Crystalline toxin	20.5	3840	42,040	14.1	16.7
104	" "	19.7	3960	42,040	12.1	13.4
131	" "	22	2880	39,460	19.7, 19.7	17.7, 21.3

of an association of toxin particles with other particles. If such were true one might hope to reveal the existence of such a situation by making determinations of sedimentation coefficients at different temperatures. At high enough tem-

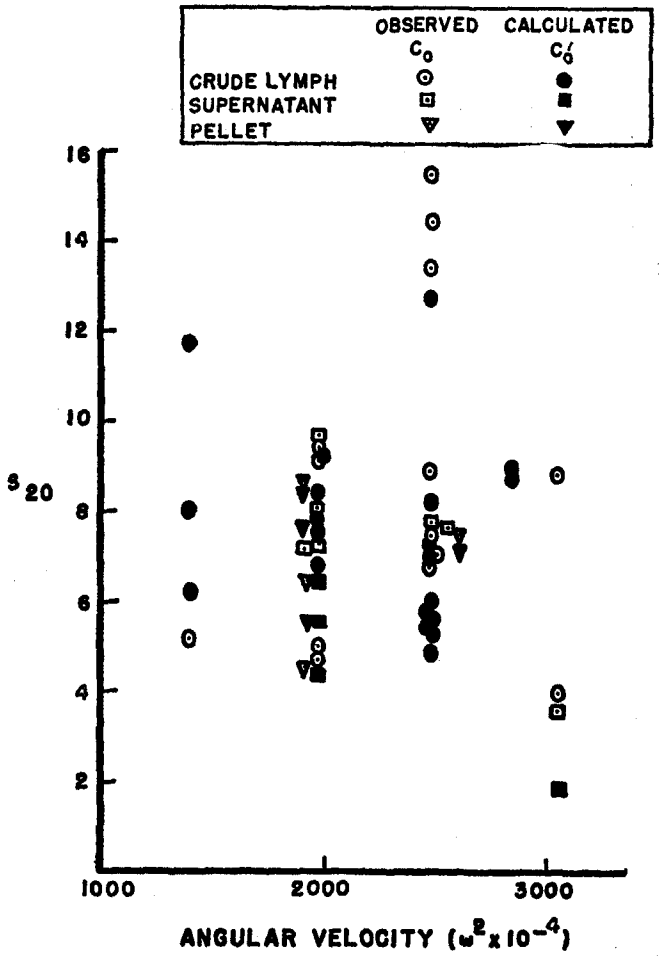

FIG. 1. A plot of the sedimentation coefficient of the botulinum toxin appearing in lymph *versus* force employed in the determination.

peratures sufficient dissociation might be expected to occur to result in decreased sedimentation coefficient values. Fig. 3 records sedimentation data obtained at different temperatures. While there seemed to be an increased variance in the observed sedimentation of toxin at the low temperatures of centrifugation, there was no indication of decreased particle size at the higher

temperatures. Thus, no evidence of temperature induced dissociation of the toxic units in lymph was found.

The occasional very high sedimentation coefficients, evident in Figs. 1, 2,

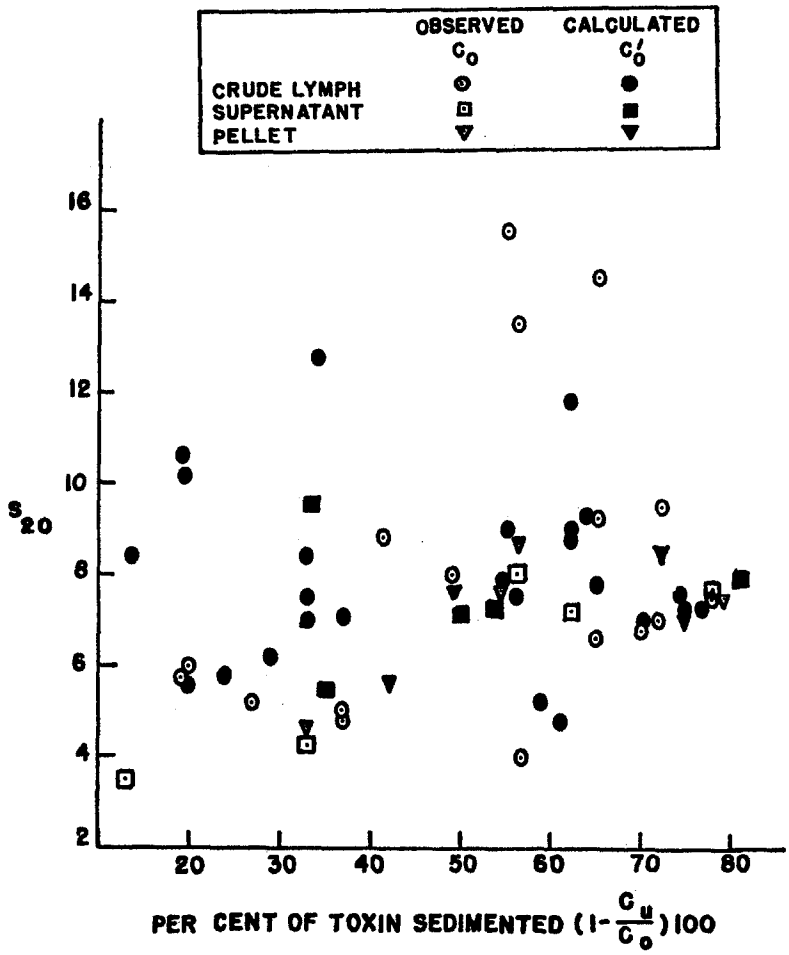

FIG. 2. A plot of the sedimentation coefficient of the toxin in lymph *versus* the percentage of toxin removed from the upper compartment of the centrifuge partition cell.

and 3 are probably not valid. By inspection of equation (1) it is clear that any loss of toxicity, either as the result of manipulative actions in making observations or by denaturation because of the low protein concentrations remaining in the upper compartment of the sedimentation cell, would result in a high apparent sedimentation coefficient. The low S_{20} values are probably due to lack of precision in the toxicity determinations alone.

The differences between the sedimentation coefficients obtained when the direct titration of the original toxicity of lymph was used for calculation, C_0 and those obtained using the calculated original toxicity, C_0' from equation

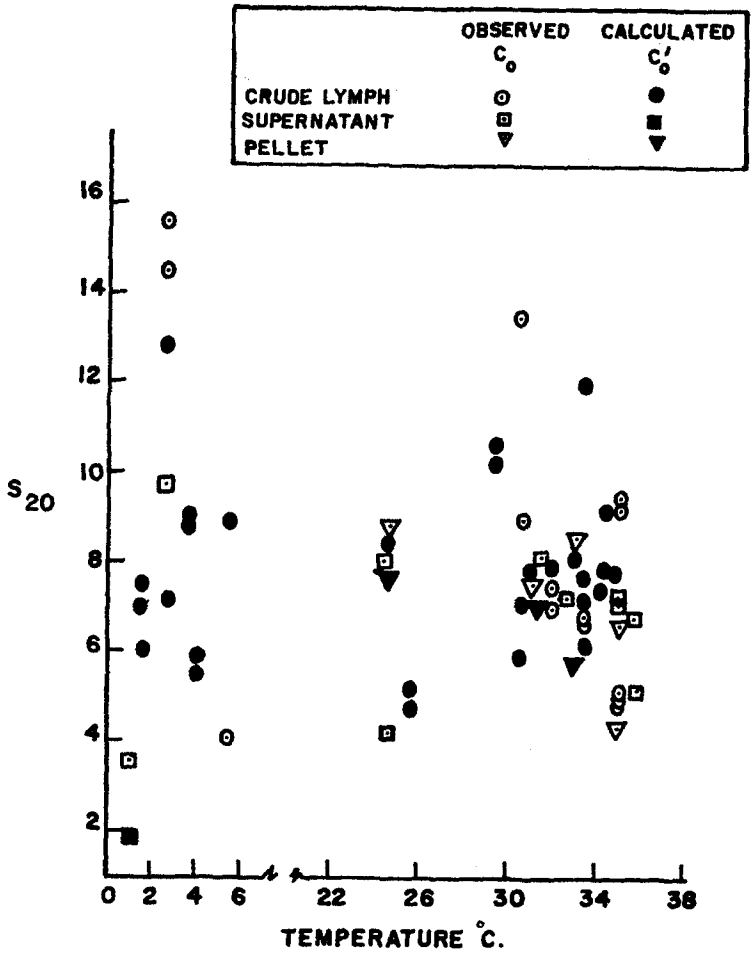

FIG. 3. A plot of the sedimentation coefficient of the toxin in lymph *versus* the temperature of the determination.

(2), can be due to a lack of precision in the toxicity titrations. As a matter of fact, the high value based on C_0 relative to C_0' is indicative of toxin inactivation accompanying the centrifugation analysis.

Fig. 4 presents the results of another method for estimating the sedimentation coefficient. This method minimizes the effect of random variation of biological assays and in general results in a more reliable estimate of the sedi-

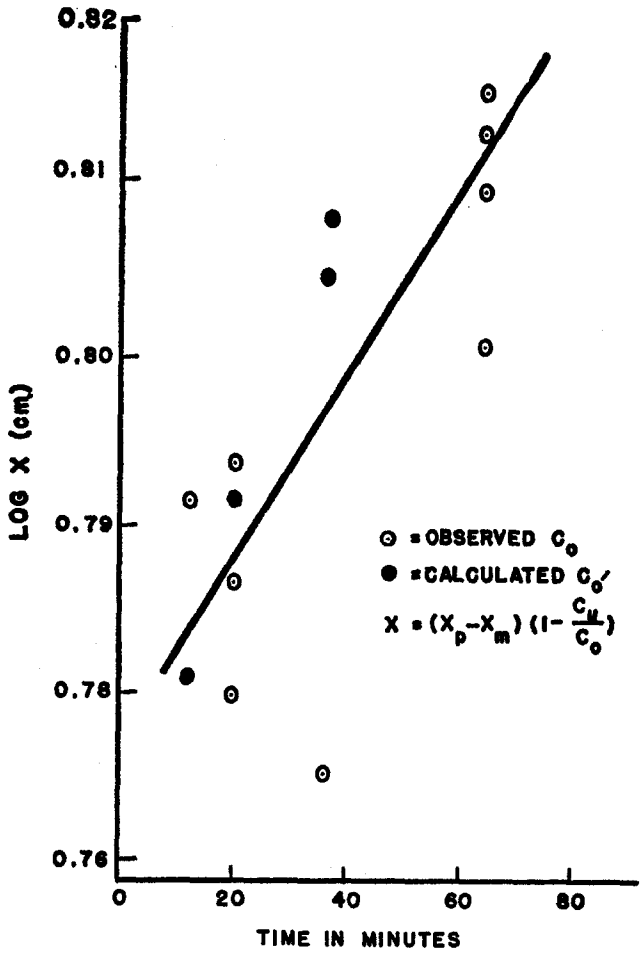

FIG. 4. A plot of toxicity *versus* time at 47,660 R.P.M. for the calculation of S_{20} according to the equation

$$S_{20} = \frac{2.303 \frac{d \log x}{dt}}{60} \left(\frac{\eta}{\eta_{20}}\right) \tag{4}$$

mentation coefficient. Using the slope of the line as drawn in Fig. 4 and equation (4) an S_{20} of 7.5×10^{-13} sec. was obtained. This is in good agreement with the bulk of the other determinations on the crude lymph recorded in Table I.

Density Gradient Centrifugation.—The results of density gradient separation of toxin in lymph in two representative trials are given in Fig. 5. The toxin tended to separate in the same way as the bulk of the protein in lymph did. The chief protein component of lymph is albumin.

For purposes of comparison it was of interest to do density gradient experiments with crystalline toxin. Figs. 6 and 7 present results representative of data obtained using crystalline botulinum toxin. For these determinations

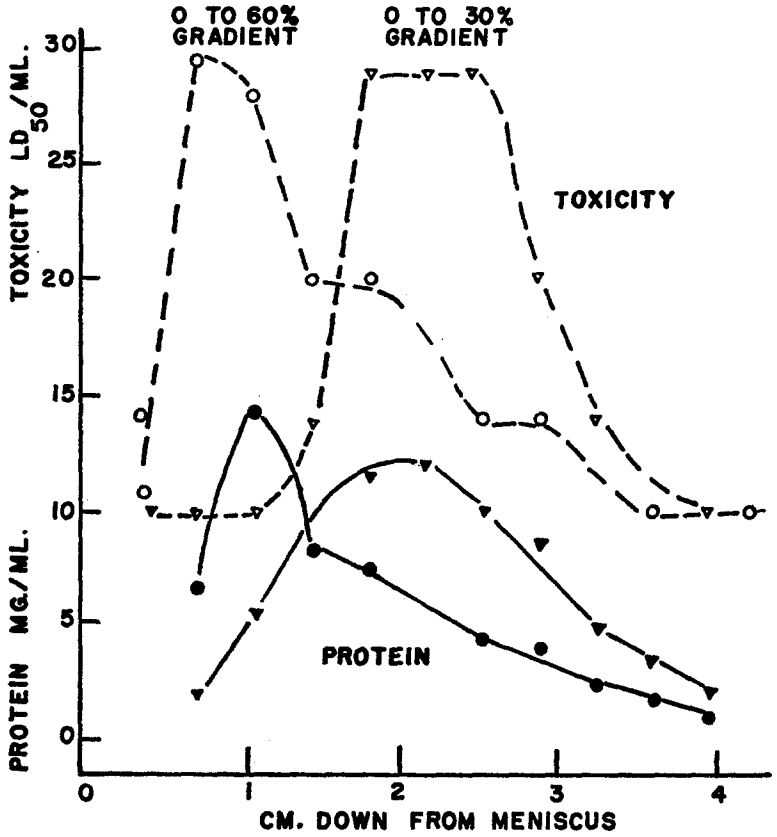

FIG. 5. Fractionation of toxic lymph after 4 hours centrifugation at 39,460 R.P.M. through density gradients. The lymph was collected from rats who had received 10 ml. of a partially purified botulinum type A toxin containing 5×10^6 mouse LD_{50} per ml. The gradients were: 10-20-40-60 and 5-10-20-30 per cent glycerol. The fractions, 0.4 ml. each, were collected at the rate of 0.2 ml. per minute.

0.2 ml. of the stock crystalline botulinum toxin or 0.2 ml. of the toxin containing 5 per cent bovine serum albumin were layered on the glycerol solutions. In 4 hours the bovine serum albumin had sedimented to the same relative position (Fig. 6) as did the principal protein in lymph (Fig. 5). In contrast, the bulk of the crystalline toxin sedimented to the bottom of the low density gradient tube in 4 hours (Fig. 6) and into the 40 per cent glycerol of the high density gradient tube (Fig. 7). It is evident by comparing Fig. 5 with Figs. 6 and 7

that the toxic unit in lymph either had a considerably smaller particle size than that of the crystalline toxic protein or was significantly less dense. Since density is a function of the amino acid composition one would not expect the density of toxin in lymph to be appreciably different from the crystalline toxin. It is most likely that the reason the toxin in the lymph had not sedimented to the

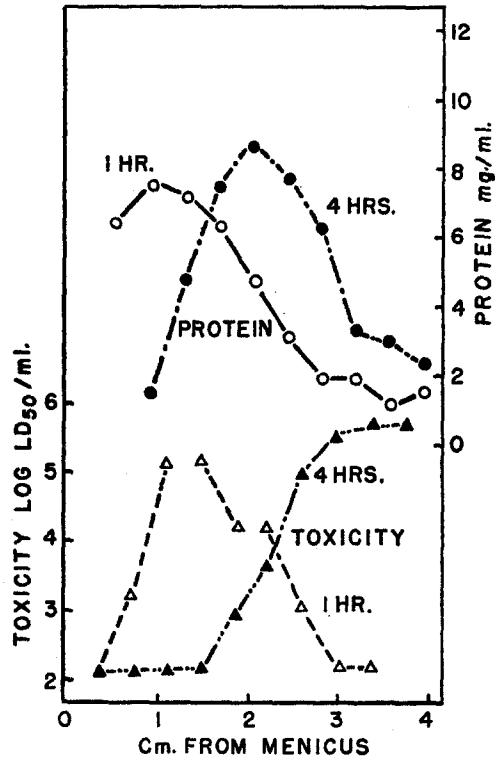

FIG. 6. Centrifugation and fractionation of a mixture of crystalline botulinum toxin and serum albumin using a low density gradient. 0.2 ml. of a 5 per cent bovine serum albumin solution containing about 10^6 mouse LD_{50} per ml. was layered on top of a gradient consisting of layers, 1 ml. each, of 5-10-20-30 per cent glycerol.

same extent as crystalline toxin was that the particles were sufficiently small not to reach an equilibrium density in the 4 hours of centrifugation.

The results of these studies using the density gradient separation system are in general agreement with the sedimentation characteristics of toxin in lymph obtained using the analytical partition cells.

Electrophoresis.—The possibility that the toxin in lymph passed the intestinal wall as a toxophore which subsequently combined with protein in lymph to yield the particles having sedimentation coefficients of about 8 Sved-

berg units was considered. Free boundary electrophoretic separation and subsequent fractionation showed that the toxin was not associated with the albumin in lymph (Fig. 8). However, the results shown in Fig. 8 are not entirely consistent. In the ascending limb the toxin moved at a rate near that of α-globulin while in the descending limb it was closer to the rate for γ-globulin.

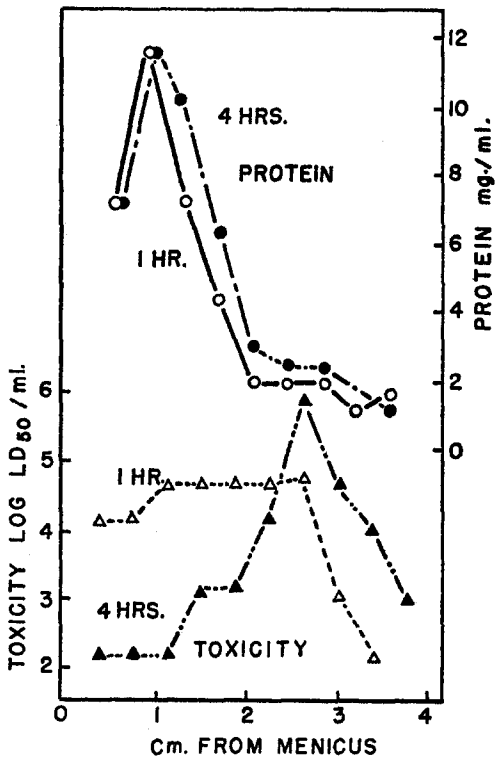

FIG. 7. Centrifugation and fractionation of a mixture of crystalline botulinum toxin and serum albumin using a high density gradient. 0.2 ml. of a 5 per cent bovine serum albumin solution containing about 10^6 mouse LD_{50} per ml. was layered on top of a gradient prepared by layering, 1 ml. each, of 10, 20, 40, and 60 per cent glycerol into the centrifuge tubes.

The ascending and descending mobilities of the toxin were about -4 and -2.5×10^{-5} cm.^{2} per volt sec. respectively. These results would indicate that the toxin was either not electrophoretically homogeneous or that the toxin was adsorbed to all of the globulins to some extent. Unfortunately a precise measurement of toxicity in the lymph fractions was not obtained and it is possible that the disparity was more apparent than real. Because all of the mice died which received the highest dilution tested it is possible that the toxicity of

fractions 9, 10, 11, and 12 of the ascending limb was actually much higher than indicated in Fig. 8.

Although the toxin in lymph need not have the same charge density as crystalline toxin it was of interest that Abrams *et al.* (8) have reported crystalline botulinum toxin to have an electrophoretic mobility of about -2.5×10^{-5}

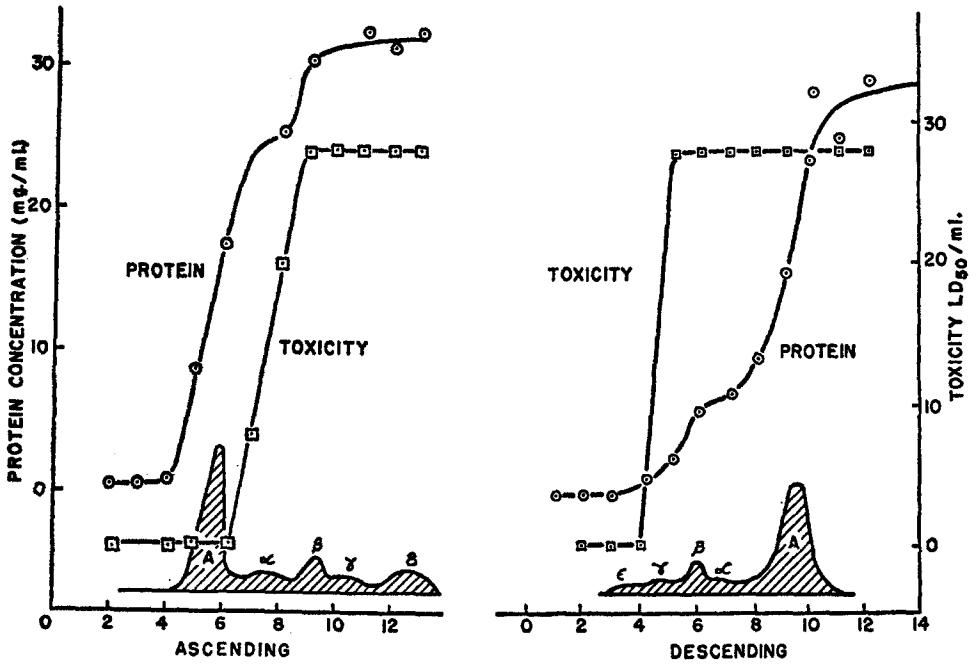

FIG. 8. Electrophoresis of toxic lymph in 0.1 M phosphate buffer at pH 7.0 for 280 minutes at 4.7 volts per cm. The schlieren pattern is shown below and on the same scale as the toxicity and total protein curves. The fractions, 0.4 ml. each, were obtained by displacing the contents of each limb at the rate of 0.2 ml. per minute.

cm.^{2} per volt sec. at pH 7, a value in excellent agreement with our observed mobility of the toxin in lymph.

DISCUSSION

The evidence presented points to the presence in lymph draining the small intestine of the poisoned rat of toxin with the dimensions of a protein. The S_{20} values of systemically absorbed toxin found in lymph had a 95 per cent confidence interval of 4.4 to 11.4 with a mean value of 7.9. These values are considerably lower than the S_{20} value of 17.3 reported for crystalline toxin (Putnam

et al. (9)) and the value of 17.9 we have found for the crystalline material employed in the present study. It is known that the toxin is associated with a separable hemagglutinin (10, 11) and Meyer and Lamanna (12) have shown the removal of hemagglutinin from toxin preparations to increase the diffusion coefficient of toxin, an indication of a smaller particle size. It has also been reported by Wagman (13) that under suitable conditions of acidity and ionic strength toxic material with an S_{20} value of 6 to 7 can be detected in crystalline toxin. The crystalline toxin employed in the present study also appears to possess a small amount of toxic material of a relatively small size since the data in Fig. 7 show some toxin sedimenting only as far as albumin in a mixture of toxin and bovine serum albumin. It would appear, therefore, that intestinal permeability is such as to favor the passage of smaller sized toxic protein appearing in crystalline toxin. Unfortunately, the concentration of toxin appearing in lymph has been insufficient to permit us to assay for hemagglutinin which we might expect to be absent and thus account in part, if not entirely, for the smaller size of toxin in lymph than crystalline toxin.

The data indicate that the toxin in lymph has dimensions within the range for proteins and need not be broken down to small peptides in order to pass through the intestinal barriers into the lymphatic system. While the observed sedimentation coefficient of the toxin in lymph was significantly less than that of crystalline toxin, it is not necessary to postulate any breakdown of the toxin in the intestine in order to account for systemic absorption. It is possible that a small percentage of the crystalline toxin was already of the smaller particle size, and it was only this portion of toxin whose passage into the lymph was favored.

There is no evidence that crystalline toxin in the intestine is broken down into smaller sized toxic particles. We base this statement on sedimentation coefficients determined for crystalline toxin both before and after exposure to residence in the small intestine for a period of 2 hours (Table II). The S_{20} values of Table II show that the particle size of the bulk of the toxin was not significantly reduced by exposure of the crystalline toxin to the digestive processes in the intestine. The sedimentation coefficient of the crystalline toxin was in good agreement with the 17.3 value reported by Putnam *et al.* (9).

We see no necessity to invent the hypothesis that the toxin crosses the barriers of the small intestine as smaller than protein particles which are reaggregated in lymph to the dimensions of a protein. Rather, we subscribe to the view that the small intestine does not present an absolute barrier to the passage of protein, and that botulinum toxin is but one among many whole proteins which are systemically absorbed from the small intestine in small quantities.

We doubt the possibility that the true particle size of toxin in lymph is inaccurately portrayed by sedimentation studies because of toxin adsorption to

albumin, the major protein component of lymph. Since albumin is the most highly charged of the lymph proteins one would expect most substances to be adsorbed above pH 7 to albumin rather than the globulins. The fact that the toxin present in lymph migrates electrophoretically at the same rate as crystalline toxin, rather than at a rate corresponding to some intermediate value, argues against the existence of a small molecular weight toxophore adsorbed to albumin. In addition, we have found that toxic lymph when dialyzed against serum albumin does not release toxic material able to pass across the walls of dialysis tubing. We conclude that the sedimentation coefficients of toxin in lymph as measured are values for toxin unassociated with albumin.

SUMMARY

On the basis of toxicity assays using partition centrifugation cells it was established that the sedimentation coefficient of type A botulinum toxin appearing in the lymph of orally poisoned rats was 7.9×10^{-13} cm. per dyne sec. with 95 per cent confidence limits of 4.4 to 11.4×10^{-13} cm. per dyne sec. This is a significantly lower value than that obtained for crystalline toxin but is well within the range of size for proteins.

Exposure of crystalline toxin for 2 hours to digestive processes in a section of the duodenum of living rats did not significantly reduce the sedimentation coefficient of the toxin. The S_{20} of crystalline toxin employed in the present study ranged between 12 and 21 with a mean value of 17.9.

While it was observed that both botulinum toxin and albumin sedimented from lymph in glycerol gradient tubes to essentially the same level no evidence was developed to indicate association between toxin in lymph and serum albumin. The electrophoretic mobility of toxin in lymph is like that of crystalline toxin and not albumin. Dialysis of toxic lymph against serum albumin does not result in the appearance of toxin in the dialysate.

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