ACTIVITY OF TYPE A BOTULINAL TOXIN AND HEMAGGLUTININ EXPOSED TO PROTEOLYTIC ENZYME[1]

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The question of the response of the toxins of *Clostridium botulinum* to proteolytic enzymes has been inspired by the oral toxicity and protein nature of these toxins. Older reports of the innocuousness of proteolytic enzymes for crude preparations of type A toxin are in disagreement with recent reports of detoxification of crystalline toxin by proteolytic enzymes (Halliwell, 1954). Exploration of the question preceded knowledge that the botulinal toxins are associated with separable hemagglutinins (Lowenthal and Lamanna, 1953). It is our purpose, therefore, to record observations on the biological activity of both the type A toxin and hemagglutinin upon their exposure *in vitro* to proteolytic enzymes. Exploratory study of the separation of the hemagglutinin from the toxin has also provided an opportunity to examine some preparations with varying relative contents of neurotoxic and hemagglutinating activities.

MATERIALS AND METHODS

Toxicity determinations. Two-fold dilutions of toxin solution were prepared in sterile 0.2 per cent gelatin-0.68 per cent phosphate buffer, pH 6.3. One-half ml quantities of a given dilution were injected intraperitoneally into 4 albino mice. LD_{50} values were calculated by the method of Reed and Muench (1938).

Hemagglutination determinations. Hemagglutination was determined by a spectrophotometric method (Lowenthal and Lamanna, 1953). A unit of hemagglutinating activity is the smallest amount of a solution agglutinating 50 per cent of chicken red blood corpuscles under the standardized conditions employed.

Toxin and hemagglutinin preparations. Solutions of purified toxin-hemagglutinin were prepared in an appropriate buffer following a series of alternating acid precipitations and resolutions starting with a culture of the Hall strain of *C. botulinum* type A grown in a broth medium of 0.8 per cent glucose, 3.2 per cent yeast autolysate (Albimi), 1.0 per cent lactalysate (BBL). In the purification process a selective separation of hemagglutinin from toxin did not result. Therefore preparations with varying quantities of hemagglutinin relative to toxin were prepared in exploratory selective adsorption and elution studies in which finely ground heat coagulated denatured chicken egg albumin was employed as adsorbent. This use of denatured egg albumin was suggested by the finding that protein acts as a receptor for the botulinal hemagglutinin (Lamanna and Aragon, 1956). The ratios of toxicity to hemagglutination in the preparations studied are listed in table 1. In crystalline toxin this ratio is of the order of 10,000. The selective adsorption-elution procedures employed do succeed in separating hemagglutinin from toxin but unfortunately also cause some detoxification. The result is the preparation of material with less toxicity per mg of protein nitrogen than is true for crystalline toxin in spite of the lower relative content of hemagglutinin. The best preparations employed in the present study had about half the potency (LD_{50}/mg N) of crystalline toxin.

Protein nitrogen determinations. Nitrogen content of preparations was estimated either by the Folin-Ciocalteau method (Herriott, 1941) or by micro-Kjeldahl analysis.

Enzyme preparations. Commercially available crystalline pepsin, trypsin, chymotrypsin, papain, and ficin were employed. A purified aminopeptidase from the "okra" strain of type B *C. botulinum* was supplied by Dr. R. C. Millonig

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TABLE 1

Toxicity and hemagglutinating activity of solutions of type A botulinal toxin of differing purity after varying periods of exposure to proteolytic enzymes

Enzyme	Toxin Employed in Trial				Exposure to Enzyme			
	Amount	Toxicity	Hemagglutinin	Ratio of LD_{50} to hemagglutinin	Time	pH	Activity related to control*	
							Toxicity	Hemagglutinin
units/mg toxin N	*mg N/ml*	*LD_{50}/ml $\times 10^6$*	*units/ml*		*hr*			
Trypsin								
0.035	0.262	0.059	1690	35	0	7.2	88	78
					24	6.6	50	93
3.7	0.026	0.016	188	85	0	7.0		102
					9			122
					24	6.7		103
2.2	0.0042	0.29	60	4,900	0	7.3	137	79
					24	6.6	42	100
29.2	0.055	1.0	172	5,800	0	6.6	100	89
					24	7.2	61	94
1.15	0.276	12.0	486	25,000	0	7.0	100	94
					30	7.2	30	118
10.9	0.023	2.4	82	29,000	0	7.2	230	99
					3		7	107
					24	6.5	<8	84
2.9	0.13	6.5	21	310,000	0	7.0	87	
					9		59	
					24		31	
9.3	0.001	0.118	<2	1,500,000	0	7.3	89	
					24	6.6	<13	
4.65	0.002	0.146	<2	24,000,000	0	7.1	110	
					24	6.6	11	
Chymotrypsin								
3.4	0.026	0.016	188	85	0	7.0		101
					24	6.9		103
0.374	0.055	1.0	172	5,800	0	6.6	75	109
					24	7.0	<6	111
Pepsin								
3.4	0.026	0.016	188	85	0	2.3		89
					3			94
					9			74
					24	2.5		74
0.41	0.276	12.0	486	25,000	0	2.0	100	110
					6		180	112
					30	2.2	94	93
12.3	0.023	2.4	82	29,000	0	2.0	83	118
					3		90	113
					6		75	93
					9		77	75
					24	1.3	70	62
1.47	0.130	6.5	21.0	310,000	0	2.0	80	
					3		91	
					9		54	
					24		150	

TABLE 1—*Continued*

Enzyme	Toxin Employed in Trial				Exposure to Enzyme			
	Amount	Toxicity	Hemagglutinin	Ratio of LD_{50} to hemagglutinin	Time	pH	Activity related to control*	
							Toxicity	Hemagglutinin
units/mg toxin N	*mg N/ml*	*LD_{50}/ml $\times 10^6$*	*units/ml*		*hr*			
Botulinal aminopeptidase								
21.2	0.026	0.016	188	85	0	7.1		55
					3			49
					24	7.1		30
3.66	0.0175	0.18	87	2,100	0	7.0	100	101
					24		8	85
0.95	0.055	1.0	172	5,800	0	6.6	120	108
					24	6.8	78	100
2.96	0.276	12.0	486	25,000	0	7.0	100	80
					30	7.2	720	91
2.78	0.023	2.4	82	29,000	0	7.2	126	73
					9	6.5	50	70
					24		57	67
4.90	0.13	6.5	21	310,000	0	7.0	94	
					3		50	
					9		17	
					24		10	
Papain								
3.26	0.026	0.016	188	85	0	6.4		93
					24	6.5		133
1.02	0.055	1.0	172	5,800	0	6.6	100	115
					24		70	101
Ficin								
3.4	0.026	0.016	188	85	0	6.3		91
					24			190
0.63	0.0175	0.18	87	2,100	0	7.7	67	107
					7			105
					24		4	89
5.2	0.055	1.0	172	5,800	0	6.6	144	119
					24		16	93
Bacillus subtilis glutamyl transferase								
0.46	0.052	0.012	128	940	0	7.8	92	83
					24	7.2	36	89
4.53	0.0053	0.32	15	2,100	0	7.4	134	115
					24	6.8	2	84
0.98	0.0245	3.0	<2	10,700,000	0	7.2	100	
					24		10	

* Biological activity is expressed as per cent of a control handled in all details exactly like the test preparation, except for the absence of enzyme, and assayed simultaneously with the test preparation.

(1956) and glutamyl transferase from *Bacillus subtilis* was obtained through Dr. R. D. Housewright. Except for the transferase the proteolytic activities of the enzymes were determined by employing casein as a substrate using optical density measurements at 280 mμ to follow proteolysis. The unit of proteolytic activity is defined as the amount of enzyme needed to digest

50 per cent of the casein in 1 ml of a 0.25 per cent solution within 15 min at 37.5 C. Sodium cyanide was used as an activator in the cases of papain, botulinal aminopeptidase, and ficin. The method of Lipmann and Tuttle (1945) was employed to estimate glutamyl transferase activity.

In each experiment in which the effect of an enzyme was tested, a control was employed which contained toxin-hemagglutinin but no enzyme. Such a control is necessary since, apart from the presence of proteolytic enzyme, experimental manipulations and conditions often result in losses of biological activity, particularly in alkaline environments in the case of toxicity. Periodically aliquots were removed from both series kept at 25 C and toxicity and hemagglutination titrations performed. A reason for conducting the tests at 25 C rather than higher temperatures was to reduce the losses of toxicity not associated with the presence of enzyme.

RESULTS AND DISCUSSION

Table 1 records the results obtained. In most cases only the data for 24-hr exposures to enzymes have been listed since determinations which were performed at shorter intervals of 3, 6, and 9 hr do not change the qualitative picture observed at 24 hr. The cases where per cent recovery of biological activity is greater than 100 are reflections of uncontrolled variations and of the fact that on occasion losses due to experimental manipulations were greater in the controls than in the series with proteolytic enzyme. They do not imply increased potency in the presence of the enzyme. One reason for such occurrences is the use of highly purified toxin in the absence of protective quantities of extraneous colloid or protein. Boor *et al.* (1955) have reviewed knowledge of this phenomenon.

Several facts are indicated by the data. Botulinal hemagglutinin is resistant to the proteolytic enzymes under the conditions of testing. To the contrary, the neurotoxin is attacked by trypsin, chymotrypsin, botulinal aminopeptidase, ficin, and glutamyl transferase from *B. subtilis* with obvious loss of biological activity. Pepsin and papain do not exhibit any striking detoxification activity. The relative proportions of enzyme-resistant hemagglutinin to enzyme-sensitive neurotoxin present in preparations do not appear to affect the outcome.

Table 2 lists reports on the effect of proteolytic enzymes on botulinal neurotoxic activity. The older reports of no detoxification by proteolytic enzymes suffer from the fact that experimenta-

TABLE 2

Reported activity of botulinal toxin in the presence of proteolytic enzyme

Enzyme and Reference	pH	Toxin Employed	Loss of Toxicity
Pepsin			
Schubel (1923)	?	Crude, type not known	None
Bronfenbrenner and Schlesinger (1924)	3	Crude, type A	None
Nelson (1927)	3	Crude, type A	None
Snipe and Sommer (1928)	3	Crude, type A	None
Chistyakov and Rodopulo (1943)	?	Crude, type A	None
Littauer (1951)	?	Crude, type A	None
Coleman (1954)	1.4	Crystalline, type A	Great
Halliwell (1954)	2.0	Crystalline, type A	Slight
Trypsin			
Schubel (1923)	?	Crude, type not stated	None
Bronfenbrenner and Schlesinger (1924)	8	Crude, type A	None
Chistyakov and Rodopulo (1943)	?	Crude, type A	None
Coleman (1954)	6.5	Crystalline, type A	Slight
Halliwell (1954)	7.0	Crystalline, type A	Great
Chymotrypsin			
Coleman (1954)	7.0	Crystalline, type A	Great
Papain			
Kalmanson and Bronfenbrenner (1943)	7.4	Crude, type A	None
Halliwell (1954)	7.0	Crystalline, type A	None

tion was limited to few titrations and few test animals, a criticism that cannot be made of the work of Coleman (1954) and Halliwell (1954). Our results are in essential agreement with the more recent findings except for the report by Coleman (1954) of extensive detoxification by pepsin.

Since unquestionably detoxification can result from the activities of trypsin and chymotrypsin, it is puzzling that botulinal toxin can act as an oral poison. It is doubtful that absorption of toxin is solely or in any significant part from the stomach where toxin would be exposed only to pepsin. May and Whaler (1958) have reviewed and added to the evidence that absorption of toxin is primarily from the intestines.

What may be significant is the persistence of some toxicity for long periods of time in the face of destruction of toxin by proteolytic enzymes. Thus in table 1 fair quantities of residual toxicity are recorded after 24 hr in spite of the destructive influence of a particular enzyme. Certainly relative to such a protein as casein the enzymes tested appear to act more slowly when the toxin is the substrate. The implication is that botulinal toxin need not be resistant to proteolytic enzymes in an absolute sense in order to act as an oral poison. It need only be digested slowly enough before complete destruction so as not to prevent a lethal quantity of toxin from passing the alimentary tract barriers. Such a situation would be in harmony with the often repeated observation of a large difference between the oral and parenteral lethal doses of botulinal toxin. From this point of view it would also be significant to know if extraneous protein as in foods can significantly slow or spare proteolytic destruction of toxicity, a point which has never been studied critically.

Another possibility is that enzymatic proteolysis of botulinal toxin involves a multistep process during which fractions of the toxin molecule can be split off prior to destruction of some sensitive specific biologically active center. Although a definitive study of this possibility has not been undertaken we doubt its existence. The variety of enzymes and chemical substances causing loss of toxicity attack different kinds of bonds characteristic of the structure of simple proteins. Thus splitting or alteration of the protein molecule in a number of different possible ways all lead to the same result. This suggests that maintenance of the structural integrity of the protein molecule as a whole is required for toxicity. Most important is that the loss of toxicity during proteolysis of a population of molecules proceeds to completion without any apparent lag in time (Halliwell, 1954).

It is probable that the toxic molecule passing the alimentary barrier is of a large size. The toxin passed into the lymph from the alimentary tract has been found by May and Whaler (1958) not to be dialyzable. Halliwell (1954) found no toxicity in the fractions of material becoming soluble in cold 0.25 per cent trichloroacetic acid following exposure of crystalline toxin to proteolytic enzymes. In addition one of us (C. L.)

TABLE 3

Diffusion coefficients and estimated molecular weights of toxin and hemagglutinin in preparations with different proportions of toxicity to hemagglutinating activity at pH 4.38

LD_{50} to Hemagglutinin Units	Toxin		Estimated Mol Wt	Hemagglutinin		Estimated Mol Wt
	Found $D_{4\ C}$	Calculated $D_{20\ C}$		Found $D_{4\ C}$	Calculated $D_{20\ C}$	
	10^{-7} cm²/sec	10^{-7} cm²/sec		10^{-7} cm²/sec	10^{-7} cm²/sec	
800,000	1.60	2.71	189,000			
>470,000	1.71	2.79	220,000			
12,100	0.81	1.32	1,800,000			
14,300	0.86	1.37	1,950,000	0.74	1.18	3,030,000
14,300	0.83	1.36	2,060,000	0.64	1.04	4,550,000

Denatured egg albumin was employed for selective adsorption-elution to prepare the test solutions which were made up in 0.1 N acetate buffer, pH 4.38 for diffusion measurements at 4 C and viscosity measurements at 4 and 20 C. Molecular weights were calculated assuming an f/f^0 ratio of 1.76 and a partial specific volume of 0.75.

has found that toxin mixed *in vitro* with pepsin, trypsin, or chymotrypsin does not result in escape of toxic material through dialysis tubing.

Although the toxin passing the alimentary barrier may be large, its molecular weight cannot be assumed to be that of crystalline toxin, that is, 900,000 (Putnam *et al.*, 1946). A smaller toxic molecule, 71,000 in estimated molecular weight, has been reported by Wagman and Bateman (1951) to dissociate from crystalline toxin at alkaline pH. Apart from environmental conditions, the degree of association between toxin and hemagglutinin must also determine the size of toxic molecules. Employing the membrane diffusion method of Northrup and Anson (1929), we have found differences in diffusion coefficient at pH 4.38 for preparations with different relative content of toxin to hemagglutinin, a smaller sized toxin molecule being associated with the relative absence of hemagglutinin (table 3).

SUMMARY

Type A botulinal hemagglutinin is not inactivated by the proteolytic enzymes tested of animal, plant, and bacterial origin. Toxicity is lost upon exposure of type A toxin to trypsin, chymotrypsin, ficin, type B botulinal aminopeptidase, and glutamyl transferase from *Bacillus subtilis*. Pepsin and papain appear not to affect toxicity appreciably. At the pH value tested, pH 4.38, the diffusion coefficient of the toxin was shown to become greater when the ratio of toxicity to hemagglutinin was increased by removal of hemagglutinin. The question of the size of the toxin molecule which passes the alimentary barrier has been discussed.

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