The Fractionation of *Clostridium welchii* ϵ -Antigen on Cellulose Ion Exchangers

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SUMMARY

A low-protein medium is described for the production of reasonable yields of *Clostridium welchii* (*perfringens*) ϵ -prototoxin. The ϵ -antigen in young cultures on this medium was virtually non-toxic, 99.8% being in the form of prototoxin. Its purity, estimated in terms of the protein content of crystalline ϵ -prototoxin, was 50%. Fractionation of concentrates of the antigen on columns of diethylaminoethyl- and carboxymethyl-celluloses suggested that a number of proteins intermediate between ϵ -prototoxin and ϵ -toxin were present which differed from the latter in their isoelectric points and in their activation ratios. The concentration and number of these intermediates increased as the incubation of cultures was prolonged. Since they could be fully converted to ϵ -toxin by trypsin it is probable that they were formed from ϵ -prototoxin by proteolytic or spontaneous degradation. In toxicity tests, death caused by unactivated antigen may be delayed. This evidence suggested that the intermediates were intrinsically non-toxic but could become activated in the animal body. The activation ratios might then be regarded as a measure of the ease with which the intermediates could be converted to toxin *in vivo*. Physical data and the amino acid composition of crystalline ϵ -prototoxin are presented.

INTRODUCTION

Of the several toxins and enzymes found in cultures of *Clostridium welchii* (*perfringens*), only two, epsilon (ϵ) and iota (ι), are known to be produced as non-toxic precursors. Epsilon toxin is produced predominantly by *C. welchii* type D and to a lesser extent by type B. There is no doubt that the enterotoxaemia and death associated with disease due to type D strains are the result of epsilon intoxication (Bennetts, 1932; Bullen & Scarisbrick, 1957). Bosworth & Glover (1934-35) were the first to show that culture filtrates of *C. welchii* type D could be 'activated', that is, their toxicity markedly enhanced, by incubation with intestinal contents. This was found to be due to tryptic activity. Turner & Rodwell (1943) extended these observations and used the term 'prototoxin' to denote the precursor. They also showed that proteases other than trypsin were capable of activating type D filtrates. More recently, Meisel, Albright & Rymkiewicz (1960) extracted a non-toxic prototoxin by ultrasonic lysis of type D vegetative organisms.

For the sake of clarity, three terms to be used in this paper may be defined: (1) ϵ -prototoxin is by definition non-toxic, and is a specific entity which becomes ϵ -toxin when it is treated with trypsin or certain other proteases. (2) ϵ -toxin is

completely toxic; its toxicity cannot be further increased by proteases. (3) ϵ -antigen is used as a general term to describe prototoxin, toxin or toxoid or a mixture of any of these. ϵ -Prototoxin, toxin and toxoid stimulate the production of antitoxin which reacts observably with them all. Differences exist in the abilities of the different antigens to stimulate antibody production and in their power to combine with antibody. These differences are usually imperceptible when the antigen is estimated by conventional flocculation with antitoxin.

Orlans, Richards & Jones (1960) worked on the purification and properties of ϵ -toxin; their purified material had an activation ratio of two, i.e. was a mixture containing equal amounts of toxin and prototoxin. The highest toxicity they obtained by activation with trypsin was 28 m.l.d./Lf. This indicates that a large proportion (80–90 %) of the purified toxin may have consisted of spontaneously produced toxoid, since a fully activated toxin usually contains 200–300 m.l.d./Lf. Verwoerd (1960) crystallized prototoxin after methanol fractionation. This is known to be a rather destructive procedure unless extreme care is taken, but the toxicities he obtained after trypsin treatment were much higher than those published by the previous authors. Activation ratios were not determined, and it is therefore impossible to establish the ratio of prototoxin to toxin in his crystals. The work of Meisel *et al.* (1960) has been repeated in these laboratories with some success. Non-toxic lysates from *Clostridium welchii* type D organisms were obtained which became toxic on activation. However, the amount of prototoxin in these lysates was very small and spontaneous activation occurred as they were being worked up (Mrs I. Batty, personal communication). The present paper describes the production of ϵ -antigen in a low-protein medium and the results obtained when concentrates were fractionated on columns of cellulose ion exchangers. The method of purification and crystallization of ϵ -prototoxin finally adopted was described in a preliminary note (Thomson, 1962). Certain properties of this crystalline material are reported here.

METHODS

Production of antigen. To facilitate purification of antigen, a low-protein medium was devised. This had the following composition: pancreatic digest of Ca caseinate, 400 ml.; yeast dialysis diffusate, 50 ml.; cyst(e)ine, 0.2 g.; glucose, 2.5 g.; thiamine, 0.5 mg.; riboflavin, 0.5 mg.; pyridoxin, 0.5 mg.; Ca pantothenate, 2 mg.; CaCl_2 , 2 g.; NaHCO_3 , 2 g.; water to 1 l. This solution was adjusted to pH 7.5 before autoclaving for 15 min. at 115°. The Ca caseinate digest was prepared by the method of Gladstone & Fildes (1940). It contained about 10–12 g. total-N and about 4 g. amino-N/l. as determined by the copper method of Pope & Stevens (1939). The yeast dialysis diffusate was prepared by dialysing a slurry of dried baker's yeast (Distillers Co. Ltd.) against water for 7 hr. at 80° in the proportions, 1.5 l. water/100 g. yeast, this diffusate being used in quantities as above.

The strain of *Clostridium welchii* type D used produced traces of α and κ toxins. No measurable quantities of λ or μ toxins were found.

Cultures were grown for 6–24 hr. in 15 l. bottles, and were harvested, after adding thiomersalate to 0.01 %, by sucking the fluid through a pad of Hyflo Supercel (Johns-Manville and Co. Ltd.) prepared on a lint base in a Buchner funnel.

Since ϵ -prototoxin is slowly activated by bacterial proteases and perhaps other factors, concentration and fractionation procedures were done at 5°. Fractions were stored at 2°.

Concentration of antigen. Culture filtrates prepared as above were concentrated by precipitation with ammonium sulphate (300 g./l.) and concentrated further by dialysis against polyethyleneglycol M.W. 15,000 ('Carbowax', Union Carbide Ltd., London, W. 1; Kohn, 1959). Seitz filtration through sterilizing (SB) mats (T. B. Ford Ltd., Loudwater, Bucks., England) removed fine particulate matter without loss of antigen.

Column fractionation. ϵ -Antigen concentrates were dialysed against appropriate buffers, applied to ion exchange columns, and fractions obtained by gradient elution. Columns were prepared with either diethylaminoethyl (DEAE)-cellulose or carboxymethyl (CM)-cellulose (DE50 and CM70, Whatman), anion and cation exchangers, respectively. For large-scale work involving about 2×10^6 Lf of antigen, 15 g. of dry ion exchanger were equilibrated with the required buffer. Fines were removed by several filtrations through lint and the remainder used to prepare a column 15 cm. $\times$ 2.5 cm. diameter. After loading with antigen, the column was eluted under gravity flow with linear pH, salt, or pH + salt gradients at a rate of 5 ml./hr./cm.² surface area. Five, ten or twenty-five ml. fractions were collected. Antigen and protein were estimated in these fractions and in pooled groups of fractions. Protein concentrations were estimated by the modified Folin-Ciocalteu method of Lowry, Rosebrough, Farr & Randall (1951) with a Hilger 'Biochem Absorptiometer' and a filter transmitting at 580 m μ .

Protein-nitrogen (protein-N). Determinations were made in triplicate by the micro-Kjeldahl method, with 5% (w/v) trichloroacetic acid as protein precipitant. Digestion with 98% H₂SO₄ was assisted by a copper/selenium catalyst. The Markham apparatus (Markham, 1942) was used for distillation.

Activation of antigens. To obtain some measure of the relative proportions of ϵ -prototoxin and toxin in different fractions, samples were activated with trypsin. For most samples taken at different stages of purification the following procedure was satisfactory. The sample was diluted in nutrient broth at pH 7.3 to 10 Lf/ml. To this was added 2% (v/v) crude ox pancreatic extract (Gladstone & Fildes, 1940) or 0.005% (w/v) crystalline trypsin and the mixture incubated for 30 min. at 37°. Activated fractions were immediately cooled and stored at 2°. Toxicity tests were usually done on the same day.

Combining power determinations. (1) *In vitro* flocculation. The flocculating capacity of activated or unactivated samples against standard antiserum was determined as follows. A series of tubes containing constant volumes of a suitable dilution of sample and a graded series of volumes of antiserum were incubated in a water bath at 50°. The neutral mixture was the first to flocculate. The combining power (flocculation) was expressed as Lf/ml. (2) *In vivo* tests. Amounts of activated antigen increasing in steps of 10% were mixed with 10 units of antitoxin and held at room temperature for 30 min. The mixtures were then injected intravenously into pairs of mice (each 18–22 g.). The mixture containing the least volume of antigen which caused death in one out of two mice within 2 days was taken as the end-point. This was the L+ dose at 10 units. Results were recorded as 10 times the reciprocal of the L+ dose at 10 units and expressed as unit equivalents (u.e.)/ml.

Toxicity tests. Determinations were made before and after activation. Samples (0.5 ml.) of a series of dilutions differing by increments of 50 % were injected intravenously into pairs of mice. The highest dilution which caused death in both mice within 4 days was taken as the minimum lethal dose (LD 100) of the sample. For ease of comparison the reciprocal of the LD 100, i.e. LD 100/ml. has been used in tabulating results.

Specific toxicity. This is defined as the ratio of LD 100/ml. (activated) to Lf/ml. of the activated sample, and is expressed as LD 100/Lf.

Activation ratio. The ratio LD 100/Lf (activated):LD 100/Lf (unactivated) is the activation ratio of the sample.

RESULTS

The results obtained at different times during growth in the casein digest medium are shown in Table 1. The Lf/mg. protein-N, which indicates the purity of the antigen in the fraction, decreased as incubation proceeded. By comparison with the purity of crystalline prototoxin, the antigen in the 8 hr. culture accounted for 50 % of the protein. Even after 120 hr., 30 % of the protein was accounted for as

Table 1. *Changes in purity, combining power and activation ratios of ϵ -antigen with age of culture of Clostridium welchii type D*

Age of culture (hr.)	Combining power		Protein-N (mg./ml.)	Lf/mg. protein-N	LD 100/ml.		Activation ratio	Specific toxicity (LD 100/Lf)
	Lf/ml.	L+ (u.e./ml.)*			Unactivated	Activated		
6	105	78	0.016	6500	42	2×10^4	500	200
24	215	—	0.042	5070	200	4.3×10^4	200	200
54	250	—	0.058	4300	250	5×10^4	200	200
120	225	195	0.056	4000	225	2.8×10^4	100	100

* u.e. = Unit equivalents.

specific ϵ -antigen. The activation ratios were much higher than those found when routine high protein media were used. After 120 hr. the activation ratio was still 100, indicating that the antigen consisted 99 % of prototoxin and 1 % of toxin. In a meat broth medium the antigen is usually fully activated after 120 hr., and even after 24 hr. the ratio is seldom more than 50. The final column in Table 1 shows specific toxicities. The value of 200 for the first three samples was normally expected during this work. After 120 hr., however, it had decreased to half this value, indicating spontaneous toxoiding during the prolonged incubation period.

Concentration and purification of ϵ -antigen. Culture filtrates were precipitated with ammonium sulphate at 300 g./l. Recoveries of 90–100 % were regularly obtained and the purity of the antigen increased by a factor of about 1.5. As expected, virtually no loss of antigen or increase in purity occurred during further concentration by dialysis against polyethyleneglycol, nor did any fraction of the polymer dialyse into the antigen concentrate. During the concentration of filtrates it was found that, despite working at 5°, a considerable decrease in activation ratios occurred. For example, a 24 hr. culture filtrate that had an activation ratio of 100 when harvested had a ratio of 10 after concentration. During the concentration procedure the specific toxicity (LD 100/unit of combining power) remained un-

changed, indicating that there was no loss in toxicity or potential toxicity. The decrease in activation ratio must, therefore, have been due to a conversion of 9% of prototoxin to toxin. With filtrates taken after shorter growth periods the conversion was much less.

Fractionation on diethylaminoethyl (DEAE)-cellulose

Concentrates from cultures grown for different periods of time were fractionated.

Growth for 24 hr. Figure 1 shows the distribution of protein obtained by gradient elution from 0.005M-Na phosphate (pH 7.0) to 0.4M-Na phosphate (pH 5.9).

Fig. 1. Fractionation of a 24 hr. culture filtrate on DEAE-cellulose equilibrated at pH 7.0 with 0.005M-Na phosphate. I. Elution with 0.005M-phosphate pH 7.0. II. Gradient elution; mixing vessel, 550 ml. 0.005M-phosphate pH 7.0, reservoir, 500 ml. 0.02M-phosphate pH 6.1. III. Reservoir, 500 ml. 0.1M-phosphate pH 5.9. IV. Reservoir, 0.4M-phosphate pH 5.9. 25 ml. fractions collected. Bulked fractions A-E.

Fractions were analysed for protein and for antigen titre (Lf) and groups of fractions were bulked as shown in Table 2, which gives the analytical data for the bulked fractions. Antigen titres followed closely the protein concentration and ϵ -antigen was present in all fractions. There was an overall recovery of antigen of 90%. Fraction A, containing the highest percentage of antigen, and having the highest activation ratio, came through with the starting buffer and was not retarded in the column. As elution proceeded, the activation ratios of fractions leaving the column decreased steadily and at the same time there was a decrease in specific toxicity, indicating that toxoid was present in the later fractions.

Growth for 8 hr. The same elution technique was used and a similar quantity of antigen was fractionated as for the 24 hr. growth. The pattern obtained for protein distribution, shown in Fig. 2, was strikingly similar to that shown in Fig. 1. Despite this, the antigen distribution was quite different. Fractions 3-6 contained 99% of the antigen applied to the column, compared with 42% in the similar fraction in

Table 2. Recovery of antigen, purities, toxicities and activation ratios of bulked fractions obtained by separation of a 24 hr. culture filtrate of Clostridium welchii type D concentrate on DEAE-cellulose. See Fig. 1

	Vol. (ml.)	Combining power		L + (u.e./ml.)*	Protein-N (mg./ml.)	Lf/mg. protein-N	LD 100/ml.		Activation ratio	Specific toxicity (LD 100/Lf)
		Lf/ml.	Recovery (%)				Unactivated	Activated		
Concentrate	40	24,000	—	21,000	3.06	7,850	1-2.5 × 10 ⁵	1-2.5 × 10 ⁶	10	40-100
Bulked A	260	1,560	42	1,380	0.131	11,900	3 × 10 ³	1.5-3 × 10 ⁵	50-100	100-200
fraction B	410	115	5	85	0.021	5,400	450	1-2 × 10 ⁴	50-100	100-200
C	280	430	12.5	300	0.036	11,900	4 × 10 ³	4 × 10 ⁴	10	100
D	490	330	17	285	0.035	9,500	3 × 10 ³	3 × 10 ⁴	10	100
E	720	170	13	114	0.053	3,200	0.7-1.7 × 10 ³	0.7 × 10 ⁴	4-10	40

* u.e. = Unit equivalents.

Table 3. Recovery of antigen, purities, toxicities and activation ratios of bulked fractions obtained by separation on CM-cellulose of an 8 hr. culture filtrate concentrate of Clostridium welchii type D after adsorption with DEAE-cellulose. See Fig. 3

	Vol. (ml.)	Lf/ml.	Recovery (%)	Protein-N (mg./ml.)	Lf/mg. protein-N	LD 100/ml.		Activation ratio	Specific toxicity (LD 100/Lf)
						Unactivated	Activated		
Concentrate	10	66,000	—	5.7	11,600	1.3 × 10 ⁵	1.3 × 10 ⁷	100	100
Bulked fractions D	115	250	4.5	0.027	9,250	1-2.5 × 10 ³	1-2.5 × 10 ⁴	10	40-100
F	50	1,340	10	0.119	11,250	2.5-5 × 10 ³	1.25-2.5 × 10 ⁵	50	100-200
H	45	900	6	0.074	12,100	2.0-4.0 × 10 ³	2 × 10 ⁵	50-100	200
K	80	2,130	26	0.182	11,700	2 × 10 ³	4-8 × 10 ⁵	200-400	200-400

Fig. 1. These fractions when bulked had an activation ratio of 100 and a purity of 11,700 Lf/mg. protein-N. They came through the column in the starting buffer.

Growth for 6 hr. Fractionation of these concentrates was similar in every way to that shown in Fig. 2; antigen was washed through by the starting buffer, its activation ratio was 500 and purity 13,000 Lf/mg. protein-N.

Fig. 2. Fractionation of an 8 hr. culture filtrate on DEAE-cellulose equilibrated at pH 7.0 with 0.005 M-Na phosphate. Gradient elution as in Fig. 1. 25 ml. fractions collected. Pooled fractions 3-6 = fraction A.

Fig. 3. Fractionation of Fraction A (Fig. 2) on CM-cellulose equilibrated at pH 6.0 with 0.005 M-Na phosphate. Gradient elution; mixing vessel, 550 ml. 0.005 M-phosphate pH 6.0; reservoir, 0.005 M-phosphate pH 7.2. 5 ml. fractions collected.

Fractionation on carboxymethyl-cellulose

Growth for 8 hr. Fraction A (pooled fractions 3–6) from the separation illustrated in Fig. 2 was concentrated with 'Carbowax' and fractionated on CM-cellulose. As seen in Fig. 3, the first fraction came through with the starting buffer. With this fraction no antigen was indicated by flocculation at any level of testing, but toxicity tests on activated samples showed traces of toxin. When estimations for protein were made on this fraction, heavy precipitation occurred with the phenol reagent, and optical densities were measured on the clear supernatant fluids after centrifuging. The cause of this interference was not pursued, but because of it one must suspect the accuracy of these measurements.

All fractions apart from the first contained considerable quantities of antigen; analytical data for the main fractions after bulking are given in Table 3. The distribution of activation ratios is of interest. As one would expect, the pattern obtained by fractionation on DEAE-cellulose (Table 2) was reversed, i.e. there was an increase in activation ratio as the pH value of elution increased; a similar increase in specific toxicity occurred. Antigen could be measured in fractions D–M, the total amount recovered accounting for 81% of that applied to the column.

Growth for 6 hr. The fraction containing the antigen obtained from DEAE-cellulose column was concentrated and applied to a CM-cellulose column. The result of pH gradient elution is shown in Fig. 4. Under the conditions used, only two fractions were obtained, the first containing no antigen and behaving like fraction A in Fig. 3. The second fraction, containing the antigen, had an activation ratio of 500, and a purity of 12,500 Lf/mg. protein-N. When this fraction was concentrated to 10^5 Lf/ml. by dialysis against 'Carbowax' the antigen precipitated as blunt needle-like crystals.

Properties of crystalline ϵ -prototoxin

Ultracentrifugal analysis. A freeze-dried preparation of crystalline ϵ -prototoxin was dissolved in and equilibrated against 0.067M-NaK phosphate (pH 7.0) and analysed in a Spinco Model E Ultracentrifuge. Use was made of the synthetic boundary cell to determine diffusion coefficient and of the Archibald technique of approach to equilibrium to determine weight average molecular weight. All runs were at 20°, and in determining the concentration the value of 0.186 ml./g. was taken as the refractive increment of the protein.

The partial specific volume, $\bar{V}$, was determined by measuring the weights of volumes of solution, solvent, and water delivered accurately with an Agla micro-meter syringe (Burroughs Wellcome and Co. Ltd., London). By this method $\bar{V} = 0.729$ ml./g.

The sedimentation coefficient corrected to water $S_{20^\circ W}$ was 2.48S at a concentration of 0.00696 g./ml. The apparent diffusion coefficients, determined from the boundary spread in a sedimentation velocity run (Schachmann, 1957), indicated that there was some decrease with time, possibly due to dependence of S_{20° on concentration. Polydispersity would be indicated by an increase of apparent diffusion coefficient with time.

The diffusion coefficient obtained from the synthetic boundary-cell run corrected to water was 6.76×10^{-7} cm.²/sec. The weight average molecular weight, determined

from approach to equilibrium data (Trautmann & Crampton, 1959), was 40,500. From these data it was possible to show that the sample of protein used contained 92.5% material of that molecular weight. The molecular weight of contaminating protein was not greater than 14,000.

From the molecular weight, partial specific volume, and sedimentation coefficient, the ratio of the frictional coefficient of the molecule to that of an unsolvated sphere, f/f_s , was calculated to be 1.68. This represents a considerable degree of asymmetry

Fig. 4. Fractionation on CM-cellulose of antigen from a 6 hr. culture filtrate not adsorbed by DEAE-cellulose. CM-cellulose equilibrated at pH 6.0 with 0.005M-Na phosphate. I. Gradient elution; mixing vessel, 550 ml. 0.005M-Na phosphate pH 6.0; reservoir, 0.005M-phosphate pH 7.0 till fraction No. 82. II. Reservoir, 0.005M-phosphate pH 7.5, 10 ml. fractions collected.

Table 4. *Amino acid composition of crystalline ϵ -prototoxin of Clostridium welchii type D*

	%		%
Lysine	14.20	Alanine	3.00
Histidine	1.28	Valine	6.95
Ammonia	2.13	Methionine	1.92
Arginine	2.55	Isoleucine	3.71
Aspartic acid	17.80	Leucine	5.80
Glutamic acid	9.95	Tyrosine	6.02
Threonine	10.00	Phenylalanine	1.72
Serine	6.07	Tryptophan	0.53
Proline	3.80	Cyst(e)ine	0.59
Glycine	3.07		

and/or hydration. Assuming a hydration of 30 %, the ratio corresponds to that of a prolate ellipsoid having an axial ratio of 9 (Onley, 1941).

Amino acid analysis. The amino acid composition of crystalline ϵ -prototoxin was determined after hydrolysis with hydrochloric acid by using a Spinco Model 120 amino acid analyser (Spackman, Stein & Moore, 1958). Cyst(e)ine was determined after oxidation to cysteic acid with performic acid by the method of Hirs (1956). Tryptophan was estimated by the ultraviolet absorption procedure of Holiday (1936). The results are given in Table 4.

DISCUSSION

By definition ϵ -prototoxin is non-toxic and becomes toxic when acted upon by certain proteolytic enzymes. There is no doubt that activation is most rapid in the presence of these enzymes but it is possible that some activation may occur slowly in their absence. In culture filtrates which contained no proteases demonstrable by azocoll digestion (Oakley, Warrack & van Heyningen, 1946) or gelatin liquefaction, there was a considerable decrease in activation ratios during incubation, and even highly purified fractions of ϵ -antigen increased in toxicity on standing. ϵ -Prototoxin may therefore be considered a labile protein in so far as its conversion to toxin is concerned, its activation, either spontaneously, or by minute traces of enzyme, being easily shown.

The adsorption of proteins by ion exchangers depends on the net charge of the protein molecule, which in its turn is a function of the pH value of the solution and the isoelectric point of the protein. In fractionations on the anion exchanger DEAE-cellulose (Fig. 1) those fractions which were eluted first had higher isoelectric points than those eluted later. This relationship between the order of elution and the isoelectric point is reversed when a cation exchanger such as CM-cellulose is used. It can be seen from Tables 2 and 3 that there was a similar decrease and increase in the activation ratios of fractions as they were eluted from DEAE- and CM-cellulose, respectively, indicating that the activation ratio varied with the isoelectric point. To extrapolate, prototoxin would have the highest and toxin the lowest isoelectric point.

The similarity between the fractionations illustrated in Figs. 1 and 2 has already been noted. The main difference was that with the 8 hr.-growth filtrate (Fig. 2) only one fraction contained ϵ -antigen, whereas the other fractions contained non-specific protein only. However, in the 24 hr. filtrate (Fig. 1) ϵ -antigen was distributed over the whole series of fractions. If the non-specific proteins were still present in cultures after 24 hr. they would be expected to appear in the equivalent fractions from the 24 hr. filtrate. This is borne out (Table 1) by the lower purities of antigen in these fractions. However, all fractions shown in Figs. 1 and 3 had some toxicity. It is difficult to explain this in terms of net charge since ϵ -toxin is assumed to be a discrete entity. Perhaps ϵ -toxin was adsorbed by these various fractions and more adsorption occurred with proteins having a lower isoelectric point. This would account for the decrease in activation ratio (or increase in toxicity/unit of combining power) of fractions as they were eluted from the column. This explanation is, however, rendered unlikely by the fact that in Fig. 2 no toxin accompanied a similar spread of fractions.

Estimations of the toxicity of ϵ -antigen fractions were performed by the intravenous injection of dilutions of the fraction into mice which were then observed for 4 days for signs of intoxication. Normally a lethal dose of ϵ -toxin from an activated filtrate will kill in less than 24 hr. and an assessment of the toxicity of activated samples can be made in 1 day. With unactivated samples, however, death is often delayed for a day or more.

The results obtained in the estimation of unactivated antigen may be explained by assuming that the injected antigen undergoes slow activation *in vivo*, the toxicity of the antigen being thus determined by the ease with which it can be activated, as well as by the amount of toxin *per se* in the antigen. The evidence obtained by ion exchange fractionation indicates that the original crude antigenic complex is composed of a number of fractions differing slightly in net charge. If these fractions are stepwise degradation products of prototoxin, that is, intermediates in the conversion of prototoxin to toxin, then the ease by which they are further degraded to toxin *in vivo* will depend upon the amount of degradation they have already undergone. The activation ratios of these fractions will therefore indicate the degree of degradation, those with the highest activation ratio being the least degraded. But it has been previously shown that the activation ratios of fractions bear a direct relationship to the isoelectric points. This means that as the prototoxin is degraded to toxin there is a gradual decrease in isoelectric point. This could be accounted for by a decrease in the number of basic groups on the molecule. The activation of prototoxin to toxin is therefore envisaged as a stepwise removal from the prototoxin molecule of basic amino acids or of peptides containing these. As the prototoxin is being activated it remains non-toxic, but the ease by which it can be converted to toxin *in vivo* is increased, the result being that there is a decrease in the activation ratios, and an increase in apparent toxicity due to *in vivo* activation of intermediates formed during activation.

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