Studies on the Preparation and Isoelectric Point of Staphylococcal α-Haemolysin

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Studies on the physicochemical properties of staphylococcal haemolysin have been hampered by the difficulty of preparing a pure, active product in sufficient quantity. Wittler & Pillemer (1948) described a three-step purification procedure which gave a product of high activity, but the results were not entirely reproducible and they did not pursue the study (L. Pillemer, personal communication). More recently, Turpin, Relyveld, Pillet & Raynaud (1954) described a four-step procedure which resulted in a material of high toxicity. Even this material was still a complex mixture, however, as shown by serological analysis. Both these methods were tried but gave unsatisfactory results when filtrates of other strains were used. The work described here was an attempt to fractionate α-toxin preparations by the use of ammonium sulphate, ethanol and Cellosolve. The isoelectric point was measured on toxin which had been further separated by electrophoresis.

MATERIALS AND METHODS

Filtrates

A strain of *Staphylococcus aureus*, designated A99 and isolated by Dr E. Levy from a human lesion, was grown in a modified Walbum medium. This consisted of Difco heart-infusion broth to which 0·2% of KH_2PO_4 and 0·03% of $MgSO_4,7H_2O$ were added. The solution was then treated with 1% (w/v) of activated charcoal for about 1 hr., filtered and the pH adjusted to 7·4 with NaOH. It was dispensed in 100 ml. quantities in 16 oz. flat bottles, and each bottle was inoculated with 0·5 ml. of an overnight broth culture of A99. The cultures were incubated at 37° in a mixture of CO_2+air or oxygen (30:70), for 68 hr. in a horizontal position. The bottles were gently rocked a few times daily. The cells were removed by centrifuging in a Sharples centrifuge, and Merthiolate (thiomersal) was added to the filtrate to a concentration of 0·01%.

A filtrate was also obtained from the Wellcome Research Laboratories, Beckenham, Kent. This was derived from the Wood 46 strain grown in a medium containing horse-muscle infusion.

Fractionation procedures

Ammonium sulphate. The pH of the filtrate was adjusted to 6·5 (glass electrode) by the addition of dil. HCl or dil. acetic acid. The solution was then cooled in ice and saturated with $(NH_4)_2SO_4$. After standing overnight at 0°, the precipitate was centrifuged at 10 000 rev./min. in a Spinco model L ultracentrifuge at 0°. The precipitate from each 2·5 l. of filtrate was extracted in about 130 ml. of water. The residue was centrifuged down and discarded. The extract was dialysed at 0°, first against tap water and finally against distilled water, until free of $(NH_4)_2SO_4$ (usually about 3–4 days when dialysed into about 50 times its volume, changed two or three times each day). Any precipitate obtained by dialysis was discarded and the solution freeze-dried. Variations in this method, consisting of changes in the concentration of $(NH_4)_2SO_4$ at different values of pH, will be referred to later.

Ethanol. Ethanol fractionations were carried out on preparations obtained from precipitations with $(NH_4)_2SO_4$. A 1% (w/v) solution of the precipitate was prepared in sodium acetate, *I* 0·05, pH 7·2. The pH of the solution was 6·9. It was cooled to 0°, and the required volume of ethanol, previously cooled to −17°, was added slowly with gentle but efficient stirring, and the mixture allowed to stand at −17° overnight. The precipitate was then centrifuged down at −5°, dissolved in water, and dialysed in the same way as the $(NH_4)_2SO_4$ preparation and freeze-dried; the supernatant was cooled to −17° in preparation for further fractionation with more ethanol. In this way precipitates were obtained with 0·5, 1·0, 1·5, 2·0, 4·0 and 6·0 vol. of ethanol.

Cellosolve. Cellosolve (2-methoxyethanol) fractionation was also carried out on a solution of an $(NH_4)_2SO_4$ preparation. A 1% (w/v) solution of the preparation in 0·15 M-sodium acetate was prepared, and cooled to 0° and 1 volume of Cellosolve, previously cooled to −17°, was added slowly with gentle but efficient stirring. The mixture was stood at −17° for 6 hr. and then centrifuged at −10° to obtain the precipitate. The supernatant was cooled to −17° and a further volume of cold Cellosolve added and treated as before. The precipitates were dissolved in 0·15 M-sodium acetate and a portion of each solution was kept at 4°, whilst the remainder was dialysed in the same way as the $(NH_4)_2SO_4$ preparation and freeze-dried.

Analytical procedures

Determination of haemolytic activity. The haemolytic activity against rabbit red blood cells was determined by the method of doubling dilution in tubes, the first tube containing a dilution of 1:10. Care was taken to cause only minimal frothing when the dilutions were mixed. An equal volume of a 2% (w/v) suspension of washed rabbit red blood cells was added to each tube and the tubes were shaken and incubated for 1 hr. in a water bath at 37°. Those showing partial haemolysis were centrifuged at about 1500 rev./min. for 5 min. and the supernatants compared visually with a standard showing 50% lysis. An

estimate was then made of the final toxin dilution which would have caused 50% haemolysis. The reciprocal of the dilution was taken to be the number of haemolytic units/ml. of the undiluted toxin solution. Whenever possible, the red blood cells were collected from the same rabbit in order to overcome variations in red-cell susceptibility. When another rabbit had to be used, the new cells were compared with those from the original. The cells were washed in 0·15 M-NaCl before use and stored in the packed state at 4°. They were discarded after 1 week.

Determination of lethal activity. The lethal activity was determined by injecting 0·5 ml. portions of appropriate dilutions of toxin, dissolved in 0·15 M-NaCl, intravenously into Swiss white mice of approx. 20 g. weight. After 24 hr. the LD_{50} was recorded. Two mice were used for each dilution for economy and it was found that they gave sufficiently reliable results.

Agar-diffusion analysis. A modification of the Elek (1948, 1949) double-diffusion-gradient method was used. Rectangular ditches (2 cm. × 0·5 cm.) were cut at right angles to each other about 0·5 cm. apart in a plate poured with filtered 2% (w/v) Noble (Difco) agar in 0·15 M-NaCl. Solutions of the toxin preparations (1%, w/v) were mixed with equal volumes of the molten-agar solution previously cooled to 56° and pipetted into one set of ditches. The other set of ditches received a 1:1 mixture of staphylococcal antiserum (Burroughs Wellcome and Co.) and agar. The plates were exposed to formaldehyde vapour and left at room temperature until the antigen–antibody lines developed.

Nitrogen determinations. Nitrogen determinations were carried out with a micro-Kjeldahl technique with hydrogen peroxide as the catalyst.

Paper electrophoresis. Paper electrophoresis was carried out in a horizontal apparatus (Franglen, 1953). Sheets of Whatman no. 1 filter paper, 24 cm. wide, were used at a voltage of 4 v/cm. for 24 hr. Sodium acetate–acetic acid, Na_2HPO_4–KH_2PO_4 and sodium barbitone–diethylbarbituric acid buffers, all at I 0·1, gave average currents of 1·1, 1·3 and 1·95 mA respectively. The toxin preparations were applied as 4% (w/v) solutions and run in quadruplicate. Dextran (6%, w/v) was applied between the toxin fractions to indicate the endosmotic flow. All runs were performed in the cold room at 4°. At the end of the run, the sheet was cut into strips and two were placed on a large plate previously poured with saline–agar containing washed rabbit red blood cells. The plate was incubated at 37° in order to determine the position of the haemolytic factor. After incubation for 45 min. the strips were removed and the plate was inspected and photographed without delay. It was found that with longer incubation diffusion occurred, which obscured the exact position of the haemolytic factor. The paper strip after removal was found to be stained with haemoglobin at the site of the haemolytic effect, but this could not be utilized as a record because of diffusion. The remaining strips were stained for protein or carbohydrate by means of bromocresol green and periodic acid–Schiff reagent respectively.

Tiselius electrophoresis. The apparatus used was described by Butler, James & Treherne (1957). Solutions of toxin (1%, w/v) dissolved in either Na_2HPO_4–KH_2PO_4 buffer, pH 6·0, 6·9, 7·46 and 8·0, or sodium barbitone–diethylbarbituric acid buffer, pH 8·7, all at I 0·075, were used. Electrophoresis was carried out with a current of 5 mA for 2–4 hr. depending on pH. Fractions were removed from the electrophoretic cell by the method of Butler & Stewart (1957). Briefly, this consists in removing very small portions from the limb of the electrophoretic cell by means of a fine needle, which is finely controlled mechanically to avoid disturbance in the fluid. The resistance of the buffer solutions was determined at 0° in a conductivity cell at 1500 cycles/sec.

RESULTS

Fractionation with haemolytic activity as the indicator. Holt (1936) precipitated staphylococcal α-toxoid by full-saturation with ammonium sulphate at pH 6·5. He mentioned that much toxin also precipitated at half-saturation. Studies were therefore made of the effect of various concentrations of ammonium sulphate at varying pH. A product prepared by precipitation with full saturation with ammonium sulphate was freeze-dried. A 1% (w/v) solution of this was made, divided into six parts and cooled in ice. A range of pH from 4·2 to 6·6 was obtained by adjusting with dilute acetic acid, with a glass electrode. Precipitates appeared which were centrifuged off in weighed centrifuge tubes. Ammonium sulphate, to give 0·25 saturation, was added slowly at 0° and the fraction collected in weighed centrifuge tubes after standing

Table 1. *Weights and haemolytic activities of fractions obtained by fractional precipitation with ammonium sulphate at varying* pH *values*

Solutions (1%, w/v) of a freeze-dried preparation were obtained by full saturation of staphylococcal filtrate with $(NH_4)_2SO_4$ at pH 6·5; pH was adjusted with dil. acetic acid. Precipitations were carried out at 0°. Initial weights, at pH 4·20, 4·50, 5·05, 5·36 and 6·61, were 100 mg.; at pH 5·94, 60 mg. Weights are expressed in milligrams, and activities in haemolytic units/mg.

Degree of saturation with $(NH_4)_2SO_4$	pH 4·20		pH 4·50		pH 5·05		pH 5·36		pH 5·94		pH 6·61	
	Wt.	Activity	Wt.	Activity	Wt.	Activity	Wt.	Activity	Wt.	Activity	Wt.	Activity
0	6·4	20	5·2	365	2·7	93	2·8	457	2·0	640	1·4	114
0·25	9·5	34	6·5	25	4·3	30	4·9	65	2·7	59	3·9	33
0·50	32·5	2400	31·2	3200	30·7	400	25·0	300	5·0	—	20·8	400
0·75	20·6	1200	27·9	1600	18·4	1600	29·5	1600	11·4	2000	19·8	1600
1·0	5·6	150	11·9	300	7·1	2500	6·4	600	5·8	500	18·0	3200

overnight. The supernatant was then treated with more ammonium sulphate and the procedure repeated. Each precipitate was dissolved in water, dialysed until free of ammonium sulphate, and freeze-dried and its haemolytic activity determined. The results are summarized in Table 1, which shows that less ammonium sulphate is required to precipitate the haemolysin at a lower pH value than at a higher: for example, 0·5 saturation at pH 4·5 gave a product of activity comparable with full saturation at pH 6·6. However, for the production of further batches, it was decided to keep to full saturation at pH 6·5 as exposure to low pH might be undesirable.

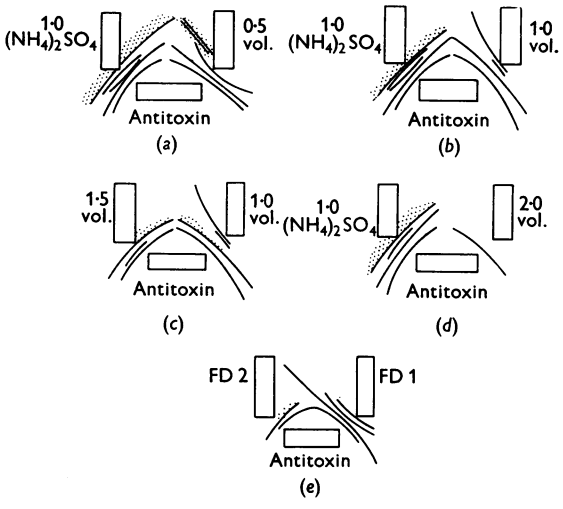

Fig. 1. Diagram of agar double-diffusion pattern obtained with fractions precipitated by ammonium sulphate, ethanol and Cellosolve. (*a*) Full saturation with ammonium sulphate [1·0 $(NH_4)_2SO_4$] and 0·5 vol. of ethanol (0·5 vol.); (*b*) full saturation with ammonium sulphate [1·0 $(NH_4)_2SO_4$] and 1·0 vol. of ethanol (1·0 vol.); (*c*) 1·5 vol. of ethanol (1·5 vol.) and 1·0 vol. of ethanol (1·0 vol.); (*d*) full saturation with ammonium sulphate [1·0 $(NH_4)_2SO_4$] and 2·0 vol. of ethanol (2·0 vol.); (*e*) 1 vol. of Cellosolve, freeze-dried (FD 1) and 2·0 vol. of Cellosolve, freeze-dried (FD 2). —, Line of antigen–antibody precipitation; ⁞⁞⁞⁞, areas of diffuse precipitation; □, antigen and antiserum ditches.

Double-diffusion plates (shown diagrammatically in Fig. 1) showed that the product obtained by saturation with ammonium sulphate consisted of at least five components, and the electrophoretic patterns were also complex (Fig. 2). Fractional reprecipitation with ammonium sulphate failed to result in increased activity, or to reduce the complexity of the double-diffusion pattern.

Ethanol fractionation of a solution of the product obtained by full saturation with ammonium sulphate gave less complex preparations, as judged by double diffusion. However, they had lower activity, and in some cases had no activity at all. The results of a typical fractionation are given in Table 2, and the diffusion patterns are shown diagrammatically in Fig. 1. Both the 1·0-vol. and the 1·5-vol. precipitates shared two lines with the parent substance, but the 1·0-vol. precipitate, which was haemolytic, had two lines unshared with the inactive 1·5-vol. precipitate. One of these might represent the haemolysin.

Fractionation with Cellosolve also gave products which were less complex, as shown by the double-

Table 2. *Haemolytic and lethal activities of fractions obtained by fractionation with ethanol*

Fractions were obtained by adding the required volume of ethanol, previously cooled to −17°, to a cold 1% (w/v) solution of a freeze-dried preparation obtained by full saturation of staphylococcal filtrate with $(NH_4)_2SO_4$ at pH 6·5, dissolved in sodium acetate, *I* 0·05.

Fraction (vol. of ethanol)	Haemolytic activity: Units/mg.	Haemolytic activity: Units/mg. of N	LD_{50} (mg.)
'Parent'	150	1028	0·05
0·5 vol.	75	—	—
1·0 vol.	88	620	0·10
1·5 vol.	—	—	>0·2
2·0 vol. 4·0 vol. 6·0 vol.	None		

Table 3. *Haemolytic and lethal activities of fractions obtained by fractionation with Cellosolve*

Fractions were obtained by adding the required volume of Cellosolve, previously cooled to −17°, to a cold 1% (w/v) solution of a freeze-dried preparation obtained by full saturation of a staphylococcal filtrate with $(NH_4)_2SO_4$ at pH 6·5, and dissolved in 0·15M-sodium acetate.

Fraction and vol. of Cellosolve	Haemolytic activity: Units/mg.	Haemolytic activity: Units/mg. of N	LD_{50}
'Parent'	200	1263	0·02 mg.
1·0 vol. (soln.*)	—	2700	0·0075 ml.
1·0 vol. (FD 1†)	800	6400	0·009 mg.
2·0 vol. (soln.*)	—	1173	0·09 ml.
2·0 vol. (FD 2†)	38	262	>0·3 mg.

* Precipitate was dissolved in 0·15M-sodium acetate.
† Precipitate was dissolved in 0·15M-sodium acetate, dialysed and freeze-dried.

diffusion technique and, in contrast with ethanol, gave a preparation with increased activity compared with the parent substance. Table 3 shows the biological activities of the substances obtained. It appears that Cellosolve is less harmful to the toxin than ethanol but it is still not selective enough to separate the toxin from the other protein material present.

Ratios of lethal/haemolytic activity. The L/H (lethal/haemolytic activity) ratios were obtained by measuring the two activities of each substance at the same time with portions taken from the same solutions. Results for the various products are shown in Table 4, and to some extent the effect of the various reagents on the biological activities can be seen. Reprecipitation with ammonium sulphate of the product originally obtained by precipitation with ammonium sulphate did not alter the ratio, but the original precipitation with ammonium sulphate and further fractionations with ethanol or Cellosolve caused marked changes. Dialysis and freeze-drying of the Cellosolve fractions also caused the ratios to increase. These results indicate that the two activities were being affected differentially by the procedures used.

Paper electrophoresis of the haemolytic factor. Although the toxin preparations were by no means pure, it was hoped that the haemolytic factor could be identified by electrophoresis and perhaps even concentrated sufficiently to enable further purification. Paper electrophoresis was used as a screening method in order to conserve material. The relationship between pH and the distance migrated (corrected for the endosmotic flow) by the haemolytic factor indicated that the haemolytic factor has an isoelectric point below pH 4·5. Strips stained for protein were very weakly coloured and no satisfactory measurements could be made. The strips did not show any polysaccharide to be present.

Tiselius electrophoresis of the haemolytic factor. When material was examined by electrophoresis in a Tiselius apparatus, followed by the determination of the haemolytic activities of the fractions removed from the cell, a different value was obtained for the isoelectric point. The schlieren pattern was complex. Some fast-moving components appeared first, which were shown to have no haemolytic activity and in subsequent runs were allowed to migrate out of the limbs. The bulk of the material stayed near the origin, but when the ionic strength of the buffer used was lowered from 0·2 to 0·075, this large peak began to split. The position of the peaks obtained at various pH values and the limits of significant haemolysis could not be correlated, and therefore it was concluded that the active factor was present in amounts too small to be detectable by the method. The instrument is capable of showing a peak at about 0·02 % of protein, so that the active factor must have been present at concentrations less than this. It was therefore decided to use the electrophoretic pattern obtained at any pH only as a check between comparable runs, particularly with different batches of equivalent preparations, as the main peaks always behaved in the same way. Fractions were then removed from the same levels in duplicate experiments. Owing to the design of the Tiselius cell, no band of activity is obtained as in zone electrophoresis, but instead the limit of migration is observed. All the experiments were so arranged that migration of the active substance occurred into the pure buffer, since the protein side of the original boundary was always active. In the figures shown, the buffer side of the boundary is on the left.

Table 4. *Comparison of lethal/haemolytic activity (L/H) ratios*

Measurements of haemolytic and lethal activities of the staphylococcal filtrates and of the various preparations obtained from them were carried out at the same time with portions taken from the same solutions.

Parent substance	Derivative	L/H ratio
1·0 sat. $(NH_4)_2SO_4$, 12/12/55	—	13·3
	0·75 sat. $(NH_4)_2SO_4$ ppt.	13·3
	1·0 sat. $(NH_4)_2SO_4$ ppt.	13·3
Filtrate, 20/4/56		5
	1·0 sat. $(NH_4)_2SO_4$ ppt.	8
Filtrate, 20/8/56		1·3
	1·0 sat. $(NH_4)_2SO_4$ ppt.	5
Filtrate, 21/9/56		2·8
	1·0 sat. $(NH_4)_2SO_4$ ppt.	6·6
1·0 sat., 20/8/56		
21/9/56		
0·75 sat. $(NH_4)_2SO_4$ ppt.		4·0
	1 vol. Cellosolve ppt. (soln.)	5
	2 vol. Cellosolve ppt. (soln.)	10
	1 vol. Cellosolve ppt. (freeze-dried)	7
	2 vol. Cellosolve ppt. (freeze-dried)	(not lethal)
1·0 sat. $(NH_4)_2SO_4$, 9/11/56		3
	1 vol. of ethanol	10

The schlieren patterns obtained by electrophoresis in phosphate buffer, I 0·075, at pH 6·0, 6·9 and 8·0, are shown in Fig. 2, together with the haemolytic activities of the fractions removed. If the fraction with 25% of the original starting activity is taken as indicating the position of the haemolytic factor, and the values of the mobilities of these fractions are plotted against pH, the position of the isoelectric point can be obtained. Fig. 3 shows the graph so obtained and the isoelectric point occurs at pH 6·4. The patterns obtained by electrophoresis at pH 6·95 in the descending limb for 261 min. are shown in Fig. 4. No activity migrating with a positive charge was

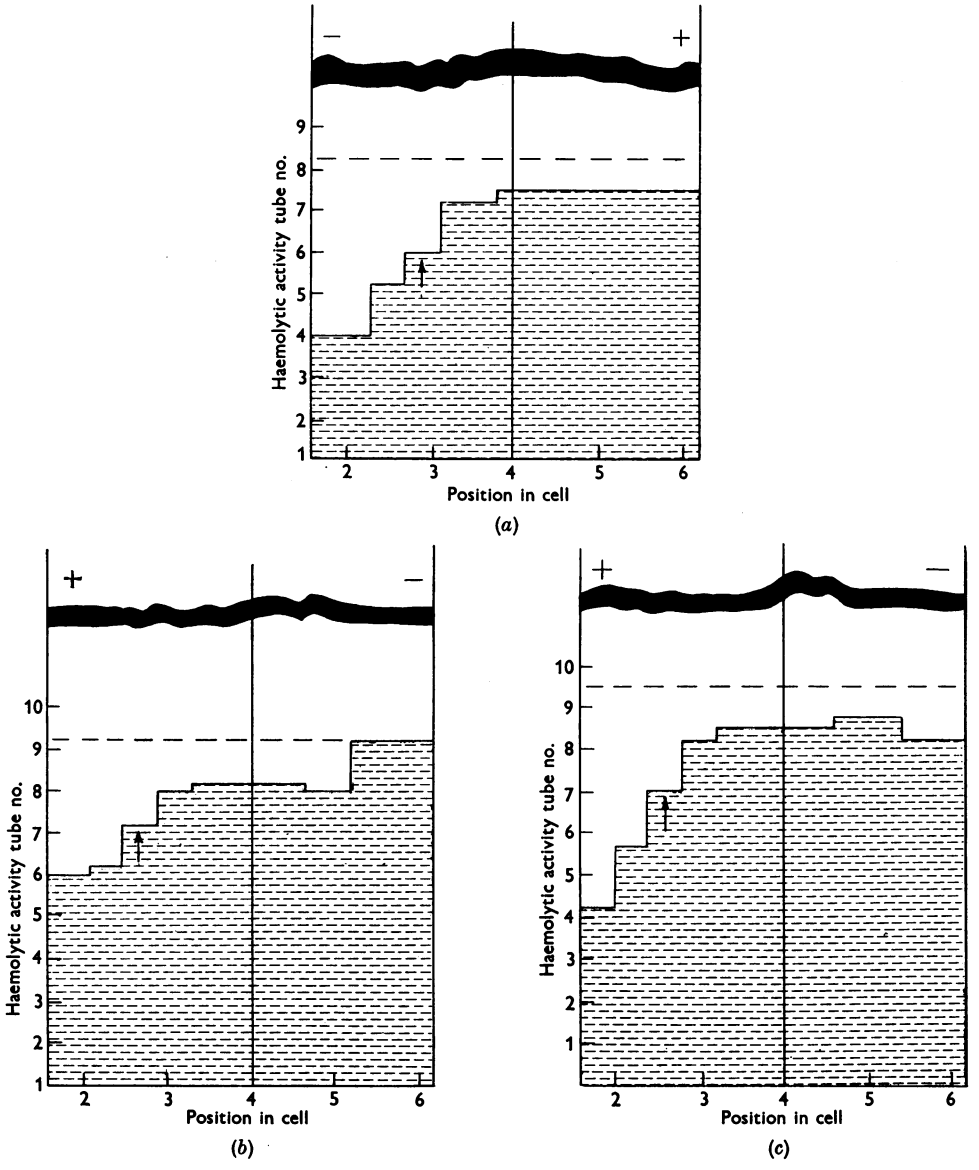

Fig. 2. Diagram of the schlieren patterns and the haemolytic activities of the fractions obtained by Tiselius electrophoresis. The continuous vertical line indicates the position of the original boundary; the broken horizontal line indicates the haemolytic activity of the original solution before electrophoresis; the arrow indicates the fraction having 25% activity. The left-hand side of the original boundary was the pure buffer side. Phosphate buffers, I 0·075. (*a*) pH 6·0, descending limb, 213 min.; (*b*) pH 6·9, ascending limb, 181 min.; (*c*) pH 8·0, ascending limb, 120 min.

obtained, i.e. the active fraction was at a pH above its isoelectric point.

It must be admitted that the mobility figures obtained may not be entirely accurate for the haemolytic factor, although this should, of course, not affect the value for the isoelectric point. The mobility figure refers to a sample of 0·12 cm.^{3} and this volume occupies a height of 4 mm. in the limb of the electrophoretic cell. This sets the limits of accuracy for the estimation. Furthermore, the 25 % haemolysis arbitrarily chosen may not be the optimum indicator effect. However, had a lower concentration been chosen the possibility of adsorption on to some other protein might have increased the error.

DISCUSSION

Chemical methods used for the purification of staphylococcal α-haemolysin have failed, probably because other materials present have properties closely similar to those of the toxin. The use of less complex media for the growth of the organism should make the fractionation methods more satisfactory. For this reason the medium described by Bramann & Norlin (1951) (a yeast diffusate–casein hydrolysate mixture) was tried, but only very low yields of toxin were obtained, and the medium was abandoned.

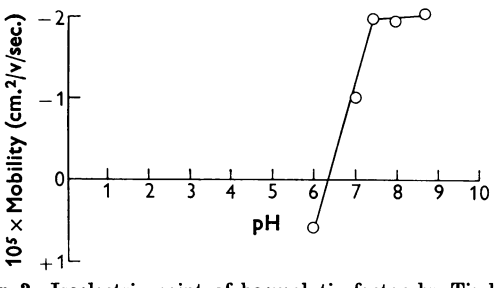

Fig. 3. Isoelectric point of haemolytic factor by Tiselius electrophoresis in phosphate buffers, I 0·075.

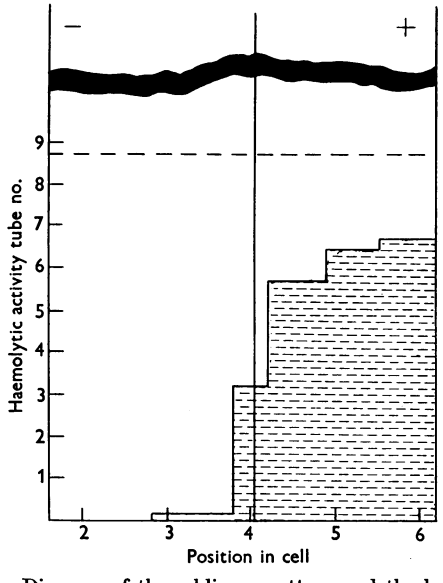

Fig. 4. Diagram of the schlieren pattern and the haemolytic activities of the fractions obtained by Tiselius electrophoresis in phosphate buffer, I 0·075, pH 6·95, descending limb, 261 min.

The differential effect of the various reagents on the haemolytic and lethal activities of the preparations suggest that the two activities are not due to the same groupings. Such differences have been noted before. Burnet (1929), although believing that the α-haemolysin and the lethal activity are due to one and the same substance, stated that the lethal effect was completely lost on the formation of a toxoid, whereas the filtrate still retained haemolytic activity. Burky (1933*a*, *b*) reported a sequence in which the lethal and haemolytic activities appeared in culture filtrates, the lethal effect appearing first, the haemolytic effect later or, in some cases, not at all. Avery, Rigdon & Johlin (1937) showed that lithium chloride had no effect on haemolysis but inhibited the dermonecrotic effect of the toxin. Whether the haemolysin and lethal effects are associated with the same or different molecules is still an open question, but the electrophoretic fractionation method may in the future be able to provide the answer. For this, a preparation of very high activity will be required, which so far is not available. Meanwhile, the electrophoretic-fractionation method does allow a limited study of the properties of the toxin.

The results obtained by paper electrophoresis indicate how misleading this method may be. The toxin appeared to run towards the cathode at any pH tested, but never as far as the dextran, thus giving the impression of being negatively charged, even at pH 5. In fact, the haemolysin must have been strongly adsorbed on to the paper, with stronger adsorption at the higher value of pH.

SUMMARY

1. Attempts to purify staphylococcal α-haemolysin by means of ammonium sulphate, ethanol and Cellosolve failed to give preparations of very high purity or activity. Ammonium sulphate was relatively harmless but limited in its powers of fractionation. Cellosolve, in contrast with ethanol, effected a further step in purification.

2. Comparison of the ratios of the lethal to haemolytic activities of the preparations indicate a differential denaturation of the active factors. Support is given for the view that there is a multiplicity of active factors.

3. The isoelectric point of the haemolytic factor was determined by an electrophoretic-fractionation method and found to be at pH 6·4.

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Studies on Gastric Proteolysis

1. THE PROTEOLYTIC ACTIVITY OF HUMAN GASTRIC JUICE AND PIG AND CALF GASTRIC MUCOSAL EXTRACTS BELOW pH 5

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Sørensen (1909), Michaelis (1914) and Northrop (1922) found that purified pepsin preparations digest proteins *in vitro* with a single maximum in the region of pH 2. Because of this, most contemporary physiological texts state that gastric proteolysis *in vivo* in mammals occurs most readily near this pH. It was observed as early as 1909, however, that gastric juice and mucosal extracts exhibited relatively greater proteolytic activity at pH 4 than did preparations of pepsin (Takemura, 1909; Hirayama, 1910), and Willstätter & Bamann (1929) reported the existence in swine gastric mucosal extracts of a second proteolytic enzyme, a cathepsin, which was not present in gastric juice and which digested gelatin and egg albumin with a pH maximum at 3·5–4·0. A proteolytic peak at this pH was also demonstrated with both Cudahy and Northrop pepsin, when acting on casein, gelatin and edestin (Dyckerhof & Tewes, 1933), and it was again suggested that a second enzyme was responsible. Norris & Elam (1940) found that crystalline salmon pepsin, when prepared by different methods, gave pH–activity curves with either one or two maxima. These observations strongly suggested that gastric mucosal extracts might exhibit two proteolytic pH maxima, one of which may sometimes disappear during the purification of pepsin.

Not until 1940 was it first demonstrated that human gastric juice may digest proteins with two pH maxima, near 2 and 4 (Freudenberg, 1940). Because activity at the second maximum could be increased by incubating with hydrogen sulphide or hydrogen cyanide, the presence of a cathepsin was again postulated. Buchs (1947) extended Freudenberg's observations, working mainly with human gastric juice on edestin, and concluded that gastric juice contained two enzymes, pepsin and cathepsin. The latter was found, unlike pepsin, to be activated by potassium cyanide, hydrogen sulphide and Co^{2+} and Mn^{2+} ions, to be relatively resistant to ultraviolet radiation and to heating at 70°, and to be inhibited by uranyl and Al^{3+} ions. Two pH maxima, near 2 and 4, have also been observed for proteolysis by human gastric juice by Merten & Ratzer (1949), Milhaud & Epiney (1951), and Bramstedt & Krüger (1954), for proteolysis by swine pepsin (Pope & Stevens, 1951; Ramer, 1954) and for proteolysis by dog gastric juice (Penitschka, 1953; Pfisterer, 1955).

In the calf also, gastric mucosal extracts have been shown to digest proteins with two pH maxima near 2 and 3·5 (Berridge, 1945). The second maximum is attributed, however, to rennin, which when crystallized from crude extracts undoubtedly digests proteins with a pH maximum near 3·5 (Berridge, 1945; De Baun, Connors & Sullivan, 1953). Rennin might thus seem to be taking the place of the 'cathepsin' of other mammals, but the absence of a second pH maximum from rennin-free extracts of calf gastric mucosa has never been demonstrated.