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# Fractionation of a Leucocidin from *Staphylococcus aureus*


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*Staphylococcus* produces at least three leucocidins, of which that originally described by Panton & Valentine (1932) is of particular interest since it attacks only white blood cells and not, like the other staphylococcal leucocidins, red blood cells as well. It is also specific to humans and rabbits. Some of the biological properties of this substance have been described by Gladstone & van Heyningen (1957). In the present paper the production of culture filtrates with high leucocidin activity is described and it is shown that this activity depends upon at least two proteins. These have been highly purified.

## MATERIALS AND METHODS

### *Culture filtrates*

*Organism.* The V8 strain of *Staphylococcus aureus* (Gladstone & van Heyningen, 1957) was used. A stock preparation on a tryptic meat–agar slope was made every month from freeze-dried organisms and cultured overnight on further tryptic meat–agar slopes to provide inocula for the production of culture filtrates.

*Comparison of media for production of leucocidin.* This was done in rocking T-tubes (van Heyningen & Gladstone, 1953) at 37° with 20 hr. growth. The cultures were clarified by centrifuging and assayed for leucocidin. Growth was estimated by suspending the organisms in 0·85% NaCl soln. and measuring their turbidity.

*Large-scale production of leucocidin.* This was done by Mr R. Elsworth and Mr R. Telling at the Microbiological Research Establishment, Porton, Wilts. The medium was that described by Gladstone & van Heyningen (1957), with the substitution of Oxoid Casamino acids and a diffusate of Oxoid yeast extract for the corresponding Difco products. Medium (80 l.) was inoculated with 20 l. of seed (prepared in turn from a 1 l. shake flask inoculated from a tryptic meat–agar slope) and the culture grown in a stainless-steel vessel with bottom aeration and stirring. When the maximum leucocidin titre was reached (8–10 hr.) the culture was cooled to below 10°, clarified by centrifuging and the supernatant sterilized by filtration. Solid $(NH_4)_2SO_4$ was then stirred in to give a 3·5M-solution and the precipitated material collected by filtration through Whatman no. 1 paper and Hyflo-Supercel. The Hyflo-Supercel and

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precipitate were scraped off the paper and extracted with 0·1 M-sodium phosphate buffer, pH 6·7. Solid $(NH_4)_2SO_4$ was added to saturate the extract and the precipitate collected by centrifuging and stored as a slurry in saturated $(NH_4)_2SO_4$ soln. This material will be known as the culture-filtrate slurry.

*Preparation of yeast diffusate.* Oxoid yeast extract (120 g.) was suspended in 85 ml. of water and dialysed in cellophan tubing (1·5 cm. × 75 cm.) against 770 ml. of distilled water for 24 hr. with mechanical agitation of both sac and diffusate. The sac developed considerable tension but rarely burst. The final diffusate contained 7·5 g. of solids/100 ml.

*Sterilization of the media.* In both the large- and the small-scale experiments medium was sterilized by autoclaving at 15 lb./in.$^2$ for 20 min. When glucose was used it was added as a sterile solution after autoclaving.

## *Assay of leucocidin*

A simple assay procedure has been developed, based on the observation of Jensen & Maaløe (1950) that leucocidin abolishes the ability of leucocytes to reduce the blue dye, phenol-indo-2:6-dichlorophenol, to a colourless compound in the presence of KCN (cf. reduction of methylene blue, Neisser & Wechsberg, 1900). Macrophages are preferred to leucocytes as they can be kept alive *in vitro* for longer periods.

Serially diluted leucocidin solutions were incubated with the macrophages, the dye and KCN were added and incubation was continued until a clear distinction could be made between those solutions which were blue (in which the macrophages were dead) and those which were white (where the dye had been reduced by the living cells). The method can be used to determine directly the leucocidin activity of a solution or its antibody-combining power.

*Minimum leucocidal dose (MLeD).* This unit is defined as the smallest amount of leucocidin in 1 ml. which will kill macrophages under these assay conditions.

*Test dose ($L^+$).* This unit is defined as the smallest amount of leucocidin in 1 ml. in the presence of 1 unit of antitoxin which will kill macrophages under these assay conditions. Solutions of leucocidin differing in concentration by 25% can be distinguished by the MLeD test, and the $L^+$ test distinguishes between leucocidin concentrations differing by 10%.

*Reagents.* (*a*) Gelatin–phosphate buffer, pH 7·2, contained (in 1000 ml.) 9·5 g. of $Na_2HPO_4$, 2·72 g. of NaCl, 5 g. of gelatin, 18·5 ml. of N-HCl and 3·6 g. of glucose. The gelatin (Harrington Bros. Ltd., London) was purified by dialysing a concentrated gel against distilled water. The dry wt. was then determined and the gels were used in the preparation of the buffer. To dissolve the gels it was necessary to boil the solution and the glucose was added after the hot solution had cooled. (*b*) Phenol-indo-2:6-dichlorophenol (referred to as the dye; British Drug Houses Ltd., Poole, Dorset) was dissolved in gelatin–phosphate buffer to give a solution with $E^{1\,cm.}_{600}$ 6·0. (*c*) KCN (110 mg./100 ml. of gelatin–phosphate buffer). (*d*) Staphylococcal antitoxin was a pepsin-refined antiserum kindly provided by Miss Mollie Barr of the Wellcome Research Laboratories. It was standardized by the method of Gladstone & van Heyningen (1957). The assays reported in this paper have been made with a single batch of antitoxin CPP 76/63.

*Macrophage suspension.* Macrophages were taken from the peritoneal cavity of rabbits 4–6 days after the injection of liquid paraffin. The suspension of macrophages in the gelatin–phosphate buffer was filtered through muslin and standardized by selection of such a concentration that 2 vol. would reduce the dye to a colourless compound under the conditions described below, whereas 1 vol. would not.

*Method.* Logarithmically falling dilutions of leucocidin were prepared in the gelatin–phosphate buffer. In the determination of the MLeD these were assayed directly; in the determination of the test dose a constant amount of antitoxin was then added and the mixtures were assayed. In most of the assays recorded in this paper the mixtures contained 0·5 unit of antitoxin/ml.

In the following stages it was found convenient to use calibrated Pasteur pipettes. A volume (2 drops) of the leucocidin dilution was withdrawn and incubated with 2 drops of macrophage suspension at 37° for 15 min. Dye (1 drop) and 1 drop of KCN were then added and the incubation was continued for 5–10 min. The end point was the tube containing the greatest dilution of leucocidin which retains the blue colour.

## *Chromatography*

*Hydroxylapatite.* This was prepared and set up in columns by the method of Tiselius, Hjertén & Levin (1956), except that the two periods of heating in mM-phosphate buffer, pH 6·7, were omitted and the material was stored in 0·1 M-sodium phosphate buffer, made by diluting the 0·5 M-phosphate buffer, pH 6·7, described below.

Fractionation of crude leucocidin on hydroxylapatite was by stepwise addition of phosphate buffers of increasing molarity. A stock 0·5 M-phosphate buffer was made by addition of NaOH to 1 mole of $NaH_2PO_4$, to give pH 6·7, and dilution to 2 l. Buffers of lower concentration were made by dilution without pH adjustment.

*Carboxymethylcellulose.* This was prepared and set up in columns by the method of Peterson & Sober (1956). Fractionation of crude leucocidin on this resin has been by gradient elution and by stepwise elution, buffers of increasing $[Na^+]$ being used at constant pH. A 0·4 M-acetate buffer, pH 5·0, was prepared by diluting 24 ml. of acetic acid and 13 g. of NaOH to 1000 ml. Buffers of lower concentration were made by dilution and buffers of higher $[Na^+]$ by addition of NaCl. In the experiments with gradient elution the $[Cl^-]$ in the effluent was determined by titration against $Hg(NO_3)_2$ with sodium nitroprusside as indicator (Kolthoff & Sandell, 1952).

*Amberlite CG-50.* This was regenerated and set up in columns by the method of Boardman & Partridge (1955). Citrate buffers were used with this resin: 0·1 M-citrate buffer, pH 5·0, contained 21 g. of citric acid, 8·07 g. of NaOH and 5·6 g. of NaCl/1000 ml.; 0·225 M-citrate buffer, pH 6·5, contained 47·7 g. of citric acid and 27·95 g. of NaOH/1000 ml.

*Fractionation with ammonium sulphate.* This was done by extracting the insoluble precipitates with $(NH_4)_2SO_4$ solutions of decreasing concentration.

## *Electrophoresis*

*Boundary electrophoresis.* This was done in an Antweiler microelectrophoresis apparatus. The buffers were those described in the section on chromatography and also 0·1 M-

sodium carbonate buffer, pH 11·2, containing 10·6 g. of $Na_2CO_3$/1000 ml., was used. With protein concentration greater than 2% it was possible to compensate the boundary before applying the electric field by slowly withdrawing fluid from one of the electrode vessels.

*Zone electrophoresis.* The method of Kunkel & Slater (1952) for zone electrophoresis in slabs of starch grains was used. Zone electrophoresis in slabs of starch gel was by the method of Smithies (1955) except that the starch gel was made from a batch of potato starch (British Drug Houses Ltd.) which had been hydrolysed in acetone–HCl for 4·5 hr. Gels containing 15% of starch had satisfactory properties.

In each case dry starch grains were added to the solution of the starting material and the suspension was inserted in a slot 0·5 cm. wide. Glucose was inserted in the slab independently and its movement during electrophoresis taken as a measure of endosmotic flow. Its position at the end of the run was determined by placing a strip of filter paper on the slab and then staining it with a $AgNO_3$ reagent (Trevelyan, Procter & Harrison, 1950).

At the completion of the run the slabs were cut in 1 cm. sections which were eluted with 0·1 M-acetate buffer, pH 5·0. The sections from the starch gel were frozen and thawed before elution.

When the crude leucocidin in 0·1 M-acetate buffer, pH 5·0, was applied to a column of starch grains it appeared with the solvent front, which was taken as evidence that adsorption was slight.

### *Analytical methods*

*Nitrogen.* This was determined by the micro-Kjeldahl method.

*Dry-weight determinations.* These were made on two samples of highly purified leucocidin, which were dialysed against distilled water, pH 5·0, for several days, centrifuged and the supernatants dried at 110° for 30 hr. The dry solid and weighing ampoule were then digested for the determinations of nitrogen.

*Ultraviolet absorption.* Measurements were made in a Unicam SP. 500 spectrophotometer.

*Protein.* Concentration of protein was assessed from the ultraviolet absorption on the assumption of an extinction coefficient $E^{0.1}_{280}$% 1·0.

*Tyrosine and tryptophan.* These were estimated from the ultraviolet absorption by use of the formulae of Goodwin & Morton (1946). No corrections were made for scatter.

*Immunological analysis.* Diffusion in agar, by the method of Ouchterlony (1949), was used.

## RESULTS

### *Development of a medium for production of leucocidin in high titre*

The 'CCY' medium employed by Gladstone & van Heyningen (1957) gave a leucocidin titre of 1–2 $L^+$/ml. Difco Casamino acids could be replaced by the corresponding Oxoid product without affecting this result. The effect of some other modifications is shown in Table 1. Reduction of the glycerophosphate concentration decreased the yield and it could not be replaced by glycerol, nor could the lactate and glycerophosphate be replaced by glucose. Substitution of Oxoid yeast extract for the corresponding Difco product gave a tenfold increase in titre. The stimulating effect of Oxoid yeast extract was maximal at 20–25%, v/v; higher concentrations were inhibitory. Fractionation of the yeast extract on ion-exchange resins suggested that the stimulation of leucocidin production may be due to several substances acting in conjunction (A. M. Woodin, unpublished results).

### *Fractionation of the culture-filtrate slurry*

*Fractionation with ammonium sulphate.* The culture-filtrate slurry was not completely soluble in 0·1 M-phosphate buffer, pH 6·7, and the solution was viscous and not suitable for application to the hydroxylapatite columns. The viscous component was absent in the fraction soluble in 1·2–2·2 M-$(NH_4)_2SO_4$, which contained 60–70% of the leucocidin, purified twofold.

This fraction was precipitated by saturation with $(NH_4)_2SO_4$. It contained diffusible substances absorbing strongly in the ultraviolet region and its protein content was assessed from the ultraviolet absorption of material which had been exhaustively dialysed against M-NaCl–0·1 M-phosphate buffer, pH 6·7.

*Fractionation on hydroxylapatite.* Preliminary experiments showed leucocidin to be adsorbed by hydroxylapatite at phosphate concentrations below 0·1 M and subsequently to be eluted when the concentration of phosphate was greater than 0·2 M.

When the product from the $(NH_4)_2SO_4$ fractionation was applied to a hydroxylapatite column a thin layer of very strongly adsorbed material was deposited on the surface. This clogging of the surface gradually decreased the flow rate and made the columns unstable. Channels frequently developed particularly when the pressure of the inflow was increased. This difficulty was overcome by dissolving the $(NH_4)_2SO_4$ product in 0·25 M-phosphate buffer, pH 6·7, and passing it through a small column of hydroxylapatite, the surface of which could be stirred from time to time. The clogging material was thus removed with complete recovery of leucocidin in the effluent, which was then diluted or dialysed to reduce the phosphate concentration to 0·1 M and applied to a fresh column of hydroxylapatite.

Fig. 1 shows the result of eluting from hydroxylapatite the crude leucocidin from the $(NH_4)_2SO_4$ fractionation. The leucocidin activity is eluted in more than one phosphate concentration range. This was not taken as evidence for fractionation of the material responsible for the activity, as the ability of a substance with a wide elution range to give rise to multiple peaks on stepwise elution from hydroxylapatite is known (Tiselius *et al.* 1956; Tiselius, 1958).

Table 1. *Effect of composition of the medium on leucocidin production by* Staphylococcus aureus

The Casamino acids and salts mixture was present in all media to give these final concentrations: Oxoid Casamino acids, 20 mg./ml.; $Na_2HPO_4,12H_2O$, 6·2 mg./ml.; $KH_2PO_4$, 0·4 mg./ml.; $FeSO_4,7H_2O$, 6·4 μg./ml.; citric acid, 6·4 μg./ml. $MnSO_4,4H_2O$, 10 μg./ml.; $MgSO_4,7H_2O$, 20 μg./ml. The pH was adjusted to 7·2–7·4 before inoculation. Cultures were in rocking T-tubes at 37° for 20 hr.

(*a*) *Effect of yeast diffusate*

Basic medium: Casamino acids and salts; sodium glycerophosphate, 20 mg./ml.; sodium lactate, 10 mg./ml.

| Additions | Growth (mg. of organisms/ml.) | Leucocidin titre ($L^+$/ml.) |
|---|---|---|
| Bacto Yeast diffusate (20%, v/v) | 10·5 | 1·25 |
| Oxoid Yeast diffusate (20%, v/v) | 8·2 | 14·0 |
| Nicotinamide (2 μg./ml.)<br>Thiamine (2 μg./ml.)<br>Cystine (500 μg./ml.)<br>Tryptophan (500 μg./ml.) | 4·0 | < 1·25 |

(*b*) *Effect of energy source*

Basic medium: Casamino acids and salts; Oxoid Yeast diffusate, 20%, v/v.

| Additions | Concn. (mg./ml.) | Growth (mg. of organisms/ml.) | Leucocidin titre ($L^+$/ml.) |
|---|---|---|---|
| Sodium glycerophosphate<br>Sodium lactate | 40<br>20 | 7·0 | 11 |
| Sodium glycerophosphate<br>Sodium lactate | 20<br>10 | 8·0 | 14 |
| Sodium glycerophosphate<br>Sodium lactate | 10<br>5 | 9·0 | 8·8 |
| Sodium glycerophosphate<br>Sodium lactate | 5<br>2·5 | 8·5 | 3·5 |
| Glycerol<br>Sodium lactate | 14·5<br>10 | 6·2 | 3·4 |
| Glycerol<br>Glucose | 14·5<br>10 | 3·5 | <2·5 |
| Sodium lactate<br>Glucose | 10<br>10 | 6·0<br>4·0 | <2·5<br><2·5 |

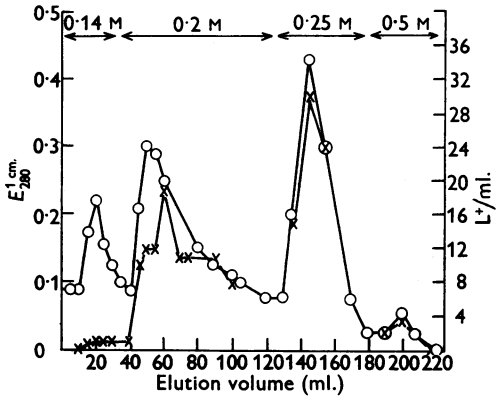


Fig. 1. Elution of crude leucocidin from a column of hydroxylapatite. A portion (142 mg.) of the $(NH_4)_2SO_4$ product from culture filtrate $P_1$, containing 2250 $L^+$ of leucocidin in 0·1 M-phosphate buffer, pH 6·7, was applied to a column 10 cm. × 1·4 cm. The horizontal arrows indicate the elution range during which the various phosphate buffers were applied to the column. ○, $E^{1\,cm.}_{280}$; ×, $L^+$/ml.

The amount of leucocidin eluted by 0·14 M-phosphate buffer was greater the smaller the column, indicating displacement effects. As extraneous protein was removed at this stage this small loss of leucocidin was not disadvantageous. In Fig. 1 there is little difference in the $L^+$/mg. of the effluent in 0·2, 0·25 and 0·5 M-phosphate buffer, pH 6·7, but with other preparations the 0·5 M-phosphate buffer effluent had less $L^+$/mg. than the 0·25 M-phosphate buffer effluent. As the purpose of the fractionation on hydroxylapatite was removal of extraneous protein, the columns were eluted with 0·14 M-phosphate buffer, pH 6·7, followed by 0·25 M-phosphate buffer, pH 6·7. This contained 40% of the leucocidin added to the columns purified two- to five-fold.

*Concentration on carboxymethylcellulose.* The eluent from hydroxylapatite in 0·25 M-phosphate buffer, pH 6·7, was adjusted to pH 5·0 with acetic acid and dialysed against 0·1 M-acetate buffer, pH 5·0. A small amount of precipitate was removed without loss of activity and the leucocidin concentrated by adsorption on a small column of

Table 2. *Properties of leucocidin preparations at various stages of purification*

With the exception of those marked with an asterisk all values are for exhaustively dialysed material.

| Culture filtrate ... ... | P1 | | P4 | | P9 | |
|---|---|---|---|---|---|---|
| Stage in purification | $L^+$/mg. of protein | $E_{280}/E_{260}$ | $L^+$/mg. of protein | $E_{280}/E_{260}$ | $L^+$/mg. of protein | $E_{280}/E_{260}$ |
| Culture-filtrate slurry | 7* | 0·9* | 19 | 1·22 | 10 | 1·20 |
| $(NH_4)_2SO_4$ product | 16* | 1·05* | 35 | 1·45 | 19 | 1·32 |
| Effluent from hydroxylapatite in 0·25 M-phosphate buffer, pH 6·7 | 65 | 1·85 | 75 | 1·80 | 42 | 1·55 |
| Product from concentration on carboxymethylcellulose | 100 | 1·95 | 80 | 1·90 | 55 | 1·75 |

carboxymethylcellulose (3 ml. of adsorbent/g. of protein). Some inactive material was unadsorbed and rejected. The column was eluted with 2 M-acetate buffer, pH 5·0, with complete recovery of the leucocidin. For storage the solution was saturated with $(NH_4)_2SO_4$. This material will be known as the 'hydroxylapatite product'.

*Comparison of different culture filtrates.* Several 100 l. culture filtrates have been processed in the way described in the preceding paragraphs. The properties of the products from the various stages are given in Table 2, and it is clear that they are not identical. Nevertheless the fractionation achieved with $(NH_4)_2SO_4$ and on hydroxylapatite is similar in all cases, and when the effluent from the hydroxylapatite columns is concentrated on carboxymethylcellulose, further inactive material is unadsorbed in each case. The ratio $E_{280}/E_{260}$ is an approximate measure of the nucleic acid content (Warburg & Christan, 1941), and reveals that nucleic acid is removed from each culture filtrate at each stage. The $L^+$/mg. is also increased in the fractionations listed in Table 2 and it is likely that this is mainly due to removal of inactive protein. It will be shown that the activity results from the synergistic action of two proteins and $L^+$/mg. values would also be increased by removal of that component which is present in excess. Separation of the component to such an extent that it was no longer in excess would be expected to result in loss of activity, and to decreased $L^+$/mg. values for protein, and so does not appear to occur at this stage.

### *Properties of the hydroxylapatite product*

*Stability.* Solutions of the hydroxylapatite product in acetate buffers, pH 5·0, were stable at 2° for several days and so were solutions in phosphate buffers, pH 6·7, where the concentration was greater than 0·1 M. At lower salt concentrations at pH 6·7, however, irreversible precipitation of some of the protein occurred with some loss of activity. Similarly, dialysis against 0·1 M-borate buffer, pH 9·0, or 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 9·0, precipitated much of the protein with irreversible loss of some of the activity. The precipitate produced in this way, at pH 6·7 or 9·0, could be dissolved at pH 10·0 but without recovery of the activity. At pH 4·0, in 0·1 M-acetate buffer, solutions slowly acquired an opalescence and irreversibily lost activity.

*Electrophoretic behaviour.* Fig. 2 records the schlieren diagrams of the hydroxylapatite product migrating in an electric field. At pH 6·0 there was little migration of any of the components. Between pH 6·0 and 8·0 the components moved very slowly to the positive electrode. At pH 5·0 all the components were positively charged and there was considerable migration to the negative electrode. Particularly at pH 5·0 the ascending and descending boundaries were not identical and the appearance suggested the formation of reversibly dissociating complexes.

Table 3 gives the result of zone electrophoresis of the hydroxylapatite product from culture filtrate P 1 in a block of starch grains. The combining power was recovered over a wide zone; 91 % of the protein, but only 50 % of the combining power, was recovered. When the fractions were mixed the resulting solution had all the combining power of the starting material. A similar result was obtained with the hydroxylapatite product from culture filtrate P 2 on electrophoresis at pH 5·0 in a slab of starch gel. With this supporting medium complete recovery of the protein from the gel was not obtained. The $L^+$/mg. of the fractions was less than that of the starting material, but when they were mixed the $L^+$/mg. of the solution was identical with that of the starting material.

### *Fractionation of the hydroxylapatite product*

*Fractionation on Amberlite CG-50.* Fig. 3 records the result of eluting the hydroxylapatite product from a column of Amberlite CG-50. When the solvent was changed from 0·1 M-citrate buffer, pH 5·0, to 0·225 M-citrate buffer, pH 6·5, a single

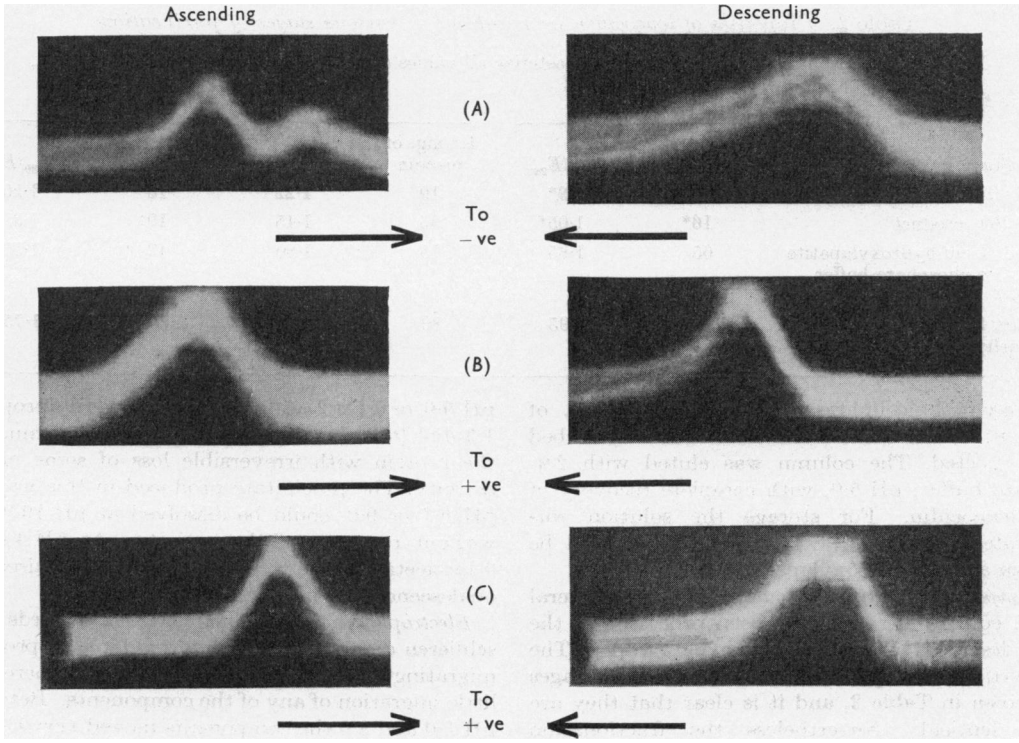


Fig. 2. Schlieren diagrams of the hydroxylapatite product from culture filtrate P9 migrating in an electric field. Protein concn., 2%. Ascending boundaries on the left-hand side. (*A*) 0·1 M-Acetate buffer, pH 5·0 (5 min. at 1·3 mA); (*B*) 0·1 M-phosphate buffer, pH 7·0 (18 min. at 2·0 mA); (*C*) 0·1 M-$Na_2CO_3$, pH 11·2 (8 min. at 2·5 mA). Starting positions at pH 5·0 and pH 11·2 were on the extreme left of the ascending-limb photographs. The boundary at pH 7·0 has moved very little.

Table 3. *Zone electrophoresis of hydroxylapatite product*

Hydroxylapatite product (42 mg.) from culture filtrate P1, containing 5000 $L^+$ of leucocidin, was added to a 0·5 cm. slot. Solvent was 0·1 M-acetate buffer, pH 5·0, current (35 mA) was passed for 20 hr. through a slab 7 cm. × 1 cm. × 30 cm. Migration was corrected for endosmosis.

| Cathodic migration (cm.) | Protein recovered (%) | Leucocidin recovered (%) | Combining power of recovered material ($L^+$/mg. of protein) |
|---|---|---|---|
| 4 | 6 | 2 | 42 |
| 5 | 22 | 10 | 58 |
| 6 | 43 | 32 | 95 |
| 7 | 20 | 6 | 80 |

trailing protein peak was observed in which the maximum combining power was not coincident with the maximum concentration of protein. The recovery of the combining power calculated from the individual volumes and combining powers of the fractions was only 20% of the input. However, when all the fractions were mixed and the mixture was assayed the recovery was quantitative. This finding, together with the electrophoretic heterogeneity, suggests that more than one substance is necessary for the activity and that they have been incompletely separated under these conditions. As it was known that the components differ little in electrophoretic mobility at pH 6·0, attempts to separate them by chromatography involving pH changes were abandoned in favour of chromatography at pH 5·0 with increasing salt concentration. On Amberlite CG-50 this is not feasible since all the components are retained by the resin even when the $Na^+$ concentration is 3·5 M.

*Fractionation on carboxymethylcellulose.* Fig. 4 records the result of eluting the hydroxylapatite product from carboxymethylcellulose with a salt gradient at pH 5·0. Again there is separation into relatively more and relatively less active fractions but recovery was only quantitative when the fractions were mixed. Further evidence that more than one substance is required for the activity was

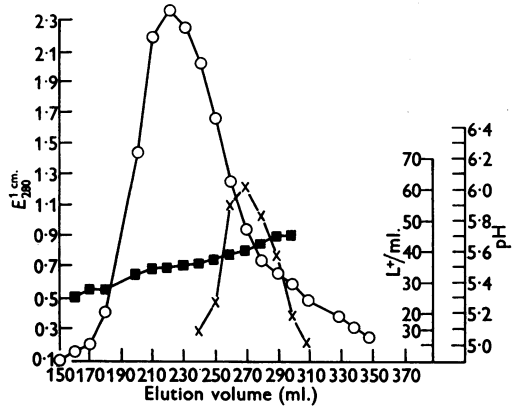


Fig. 3. Elution of the hydroxylapatite product from Amberlite CG-50. A portion (252 mg.) of the hydroxylapatite product from culture filtrate P9, containing 18 000 $L^+$ of leucocidin, in 0·1 M-citrate buffer, pH 5·0, was adsorbed on a column 11 cm. × 1·4 cm. Elution volume was measured after changing inflow buffer to 0·225 M-citrate buffer, pH 6·5. ○, $E^{1cm.}_{280}$; ■, pH; ×, leucocidin titre.

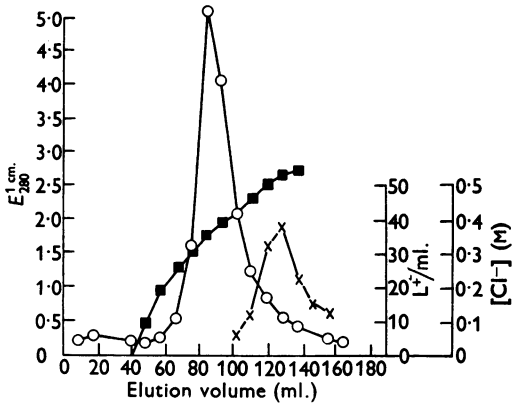


Fig. 4. Elution of the hydroxylapatite product of culture filtrate P9 from carboxymethylcellulose with a salt gradient. A portion (180 mg.) containing 6600 $L^+$ of leucocidin was added to a column 40 cm. × 0·6 cm. Gradient elution was from 0·1 M-acetate buffer, pH 5·0, to M-NaCl–0·1 M-acetate buffer, pH 5·0. ○, $E^{1cm.}_{280}$; ■, $[Cl^-]$; ×, $L^+$/ml.

obtained in the following way. In continuation of the experiment recorded in Fig. 4 the fractions eluted in the volume 66–100 ml. and those in the volume 120–190 ml. respectively were combined (i.e. those fractions eluted in the volume 100–120 ml. were omitted). These two solutions were assayed separately and then mixed together. The result, recorded in Table 4, shows clearly that there is synergism of the two fractions when they are mixed. There is little significant difference between the MLeD/$L^+$ ratio of each fraction and their mixture, which is evidence that little formation of toxoid occurs.

At lower concentrations of the faster-moving components of the hydroxylapatite product better chromatographic separation was obtained. A sample of the hydroxylapatite product was adsorbed on a column of carboxymethylcellulose and fractions were separated by stepwise addition of solvents of increasing salt concentration. The results are recorded in Table 5. Only the last fraction had significant leucocidin activity, representing 30 % of the input, and was adsorbed on a further column of carboxymethylcellulose and eluted with a salt gradient at pH 5·0. Fig. 5 records the result and shows a clear separation into two protein peaks.

These results suggested that the components of the hydroxylapatite product could be separated by a two-stage stepwise elution followed by a gradient analysis on carboxymethylcellulose. A portion (700 mg.) of the hydroxylapatite product from culture filtrate P 9 was applied to a column of 5 g. of carboxymethylcellulose and the following fractions were obtained: (1) that eluted by 0·175 M-NaCl–0·2 mM-acetate buffer, pH 5·0; (2) that eluted by 2 M-acetate buffer, pH 5·0. Then 100 mg. of (2) was adsorbed on a further carboxymethylcellulose column and eluted with a salt gradient which gave two protein peaks (Fig. 5). The two fractions eluted by stepwise elution will be referred to as displacement eluates 1 and 2. The two obtained by further fractionation of (2) by gradient elution will be referred to as the fast and slow fractions. The displacement eluate 1 and the fast and slow fractions from this experiment were each re-chromatographed on the same carboxymethylcellulose column with a salt gradient. The results of

Table 4. *Combining power of the fractions of the hydroxylapatite product*

The fractions were separated on carboxymethylcellulose in the way described in Fig. 5.

| Elution ranges (ml.) | | Volume (ml.) | $L^+$/mg. of protein | Leucocidin yield ($L^+$/ml. × volume) | MLeD/$L^+$ |
|---|---|---|---|---|---|
| 66–100 | | 35 | 1·9 | 150 | 1000 |
| 120–190 | | 70 | 80 | 1900 | 300 |
| 66–100 | Mixed | Calc. 105 | 52 | 2050 | 560 |
| 120–190 | | Found 105 | 42 | 4200 | 400 |

these three experiments show (Fig. 6) that each protein peak emerged in the same salt concentration range by which it had originally been separated. Only the slow fraction had sufficient activity to permit measurement of the test dose and these measurements suggest that this residual activity is a consequence of contamination with a small amount of the fast fraction. The fractions from re-chromatography of displacement eluate 1 and of the fast fraction were each assayed in the presence of a constant excess of the slow fraction. These assays indicate slight heterogeneity of the fast fraction and considerable heterogeneity of displacement eluate 1.

That the poor separation of the fast and slow fractions in direct-gradient analysis of the hydroxylapatite product is due to overlap of the fractions and not to their interaction was confirmed in the following way. The hydroxylapatite product from

Table 5. *Elution of the hydroxylapatite product from carboxymethylcellulose by stepwise increases in concentration of salt*

The product (210 mg.), containing 12 000 $L^+$ of leucocidin, was applied to a column 2·4 cm. × 5·0 cm.

| Eluent | Total protein eluted (%) | $L^+$/mg. of protein |
|---|---|---|
| 0·2 M-Sodium acetate, pH 5·0 0·042 M-NaCl | 6·5 | 1·15 |
| 0·2 M-Sodium acetate, pH 5·0 0·07 M-NaCl | 12·5 | 1·0 |
| 0·2 M-Sodium acetate, pH 5·0 0·175 M-NaCl | 51 | 5·2 |
| 0·2 M-Sodium acetate, pH 5·0 0·58 M-NaCl | 22 | 58·0 |

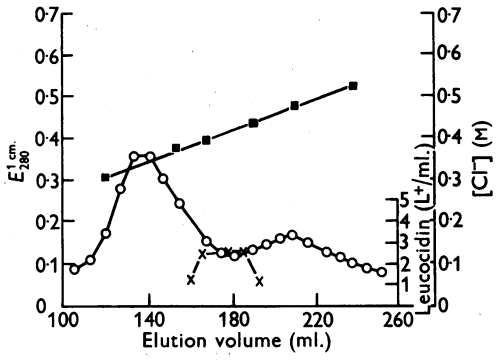


Fig. 5. Gradient elution of partially purified leucocidin from carboxymethylcellulose, demonstrating the separation into fast- and slow-moving components. A portion (26 mg.) containing 1500 $L^+$ was applied to a column 35 cm. × 1·0 cm. The gradient was from 0·1 M-acetate buffer, pH 5·0, to M-NaCl–0·1 M-acetate buffer, pH 5·0. ○, $E^{1\,cm.}_{280}$; ×, $L^+$/ml.; ■, [$Cl^-$].

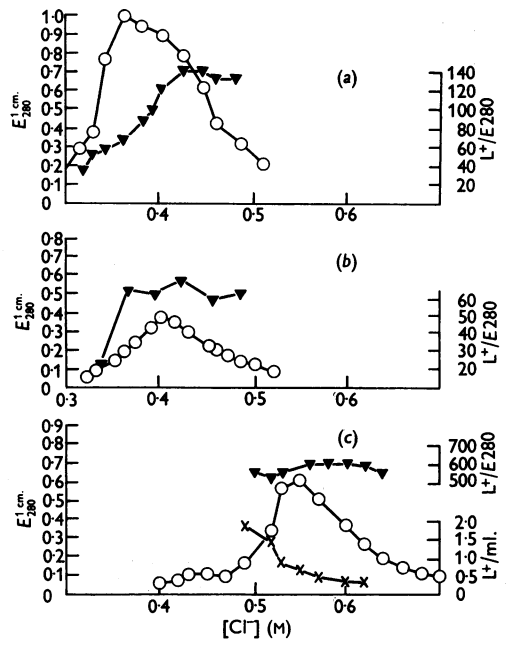


Fig. 6. Rechromatography on carboxymethylcellulose of the three fractions derived from the hydroxylapatite product. The same column of carboxymethylcellulose was used for each component with the rejection of the top 2–3 mm. of resin between each experiment. Column dimension was 35 cm. × 1·0 cm. (*a*) First displacement eluate. (*b*) Fast fraction. (*c*) Slow fraction. ○, $E^{1\,cm.}_{280}$; ▼, $L^+$ determination in the presence of second fraction (see text); ×, $L^+$ determinations without additions.

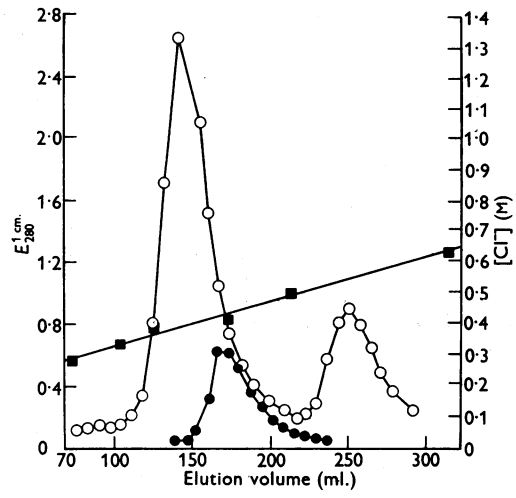


Fig. 7. Gradient elution of the hydroxylapatite product from culture filtrate P1 and rechromatography of the fast fraction after displacement separation (see text). The same column (39 cm. × 1 cm.) was used for each gradient analysis. ■, [$Cl^-$]; ○, $E^{1\,cm.}_{280}$ for hydroxylapatite product; ●, $E^{1\,cm.}_{280}$ for rechromatography of fast fraction.

culture filtrate P1 was fractionated by gradient elution on carboxymethylcellulose and the material eluted in the range 0·335–0·490 M-$Cl^-$ was adsorbed on a further column of carboxymethylcellulose and separated into two fractions by stepwise addition of 0·175 M-NaCl–0·2 M-acetate buffer, pH 5·0, followed by 2 M-acetate buffer, pH 5·0. The latter fraction was adsorbed on the same carboxymethylcellulose column used for the gradient analysis of the hydroxylapatite product and eluted with a salt gradient. There is no evidence for the appearance of two peaks and little material is eluted above 0·49 M-$Cl^-$ (Fig. 7).

*Gradient analysis of different batches of the hydroxylapatite products*

Gradient analysis of the hydroxylapatite products has shown only two components but the proportions of these have differed. Culture filtrates P1, P4 and P9 have given hydroxylapatite products with $L^+$/mg. of 100, 80 and 55 respectively (Table 2), and these contained 25, 13 and 4·5 % of the slow fraction. It is possible that the different $L^+$/mg. values are a consequence of the different ratios of the fractions but correlation must await a more precise determination of the contribution of each fraction to the total $L^+$ value.

*Properties of the components of the hydroxylapatite product*

On the basis of the preceding experiments the procedure described in Table 6 was used to prepare the displacement eluate 1 and the fast and slow fractions of leucocidin. They were stored in saturated $(NH_4)_2SO_4$.

The slow fraction of leucocidin dissolved readily in 0·1 M-acetate buffer, pH 5·0, or in 0·2 M-phosphate buffer, pH 7·0. Solutions of 3 % (w/v) concentration were clear, free from pigment and were stable for several days at 2°; on dialysis against distilled water they remained clear. Boundary electrophoresis in 0·1 M-acetate buffer, pH 5·0, or in 0·2 M-phosphate buffer, pH 7·0, showed a single component. At pH 5·0 the slow component is positively charged, at pH 7·0 it is negatively charged but migrates very slowly. Immunological analysis by the Ouchterlony method showed two bands at 0·4 % antigen concentration, of which one showed a reaction of identity with one of the bands of the fast fraction of leucocidin and was not visible at 0·1 % antigen concentration (Fig. 8). Comparison of the immunological analysis of two-fold dilutions of the slow and fast fractions of leucocidin indicated that the slow fraction was contaminated with about 3 % of the fast fraction. The slow fraction contained 15·0 % of N, 8·2 % of tyrosine, 2·2 % of tryptophan and the ratio $E_{280}/E_{260}$ was 2·15 at pH 5·0, indicating absence of nucleic acid.

The fast fraction was dissolved in 0·1 M-acetate buffer, pH 5·0, and gave solutions which at 3·5 % were slightly brown. On standing these solutions developed an opalescence and in several days deposited a precipitate. Boundary electrophoresis in 0·1 M-acetate buffer, pH 5·0, showed two peaks in the ascending limb with fusion of the two peaks in the descending limb. The schlieren diagram was similar to that of the hydroxylapatite product from culture filtrate P9 (Fig. 2). The precipitate stored in saturated $(NH_4)_2SO_4$ dissolved in 0·2 M-phosphate buffer, pH 7·0, but on dialysis against this solvent a precipitate was deposited containing 40 % of the total material. The supernatant on boundary electrophoresis at pH 7·0 showed a single peak migrating slowly to the anode. Immunological analysis by the Ouchterlony method showed three bands, a sharp band nearest the antibody well and two diffuse bands. At 0·2 % antigen concentration the diffuse bands fused. As the antigen was successively diluted the outermost diffuse band appeared increasingly nearer the antigen well but the position of the other bands was little affected (Fig. 8). The fast fraction contained 15·2 % of N, 8·4 % of tyrosine and 2·7 % of tryptophan. The ratio $E_{280}/E_{260}$ was 1·95 at pH 5·0, indicating that little nucleic acid was present.

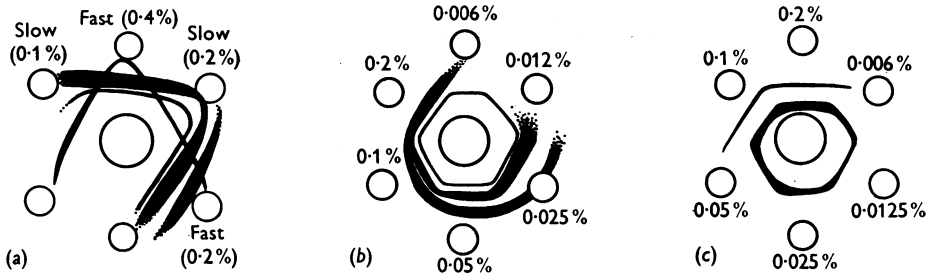


Fig. 8. Immunological analysis of the fast and slow fractions of leucocidin. In all cases the antiserum was in the centre well. (*a*) Fast and slow fractions to show the reaction of identity of the contaminant of the slow fraction with one of the bands of the fast fraction. (*b*) Serially diluted fast fraction. (*c*) Serially diluted slow fraction.

Table 6. *Fractionation of culture filtrate P 12*

P 12 contained 1 300 000 $L^+$ of leucocidin.

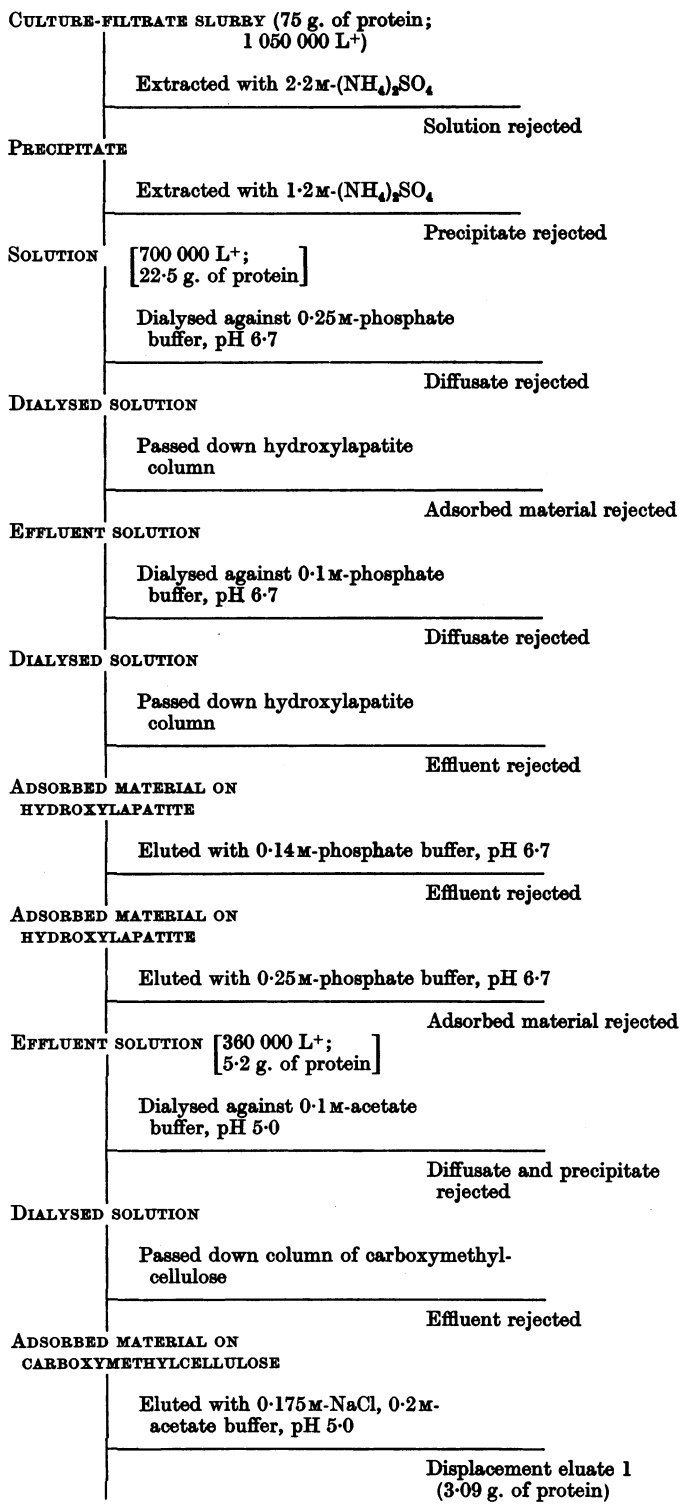

Table 6 (*cont.*)

ADSORBED MATERIAL ON CARBOXYMETHYL-CELLULOSE

Eluted with 2M-acetate buffer, pH 5·0

Adsorbed material rejected

EFFLUENT SOLUTION (590 mg. of protein)
DISPLACEMENT ELUATE 2

Diluted to give solution in 0·1M-acetate buffer, pH 5·0

SOLUTION

Passed down column of carboxymethyl-cellulose

Effluent rejected

ADSORBED MATERIAL ON CARBOXYMETHYL-CELLULOSE

Gradient applied to M-NaCl, 0·1M-acetate buffer, pH 5·0

FAST FRACTION (80 mg. of protein) | SLOW FRACTION (380 mg. of protein)

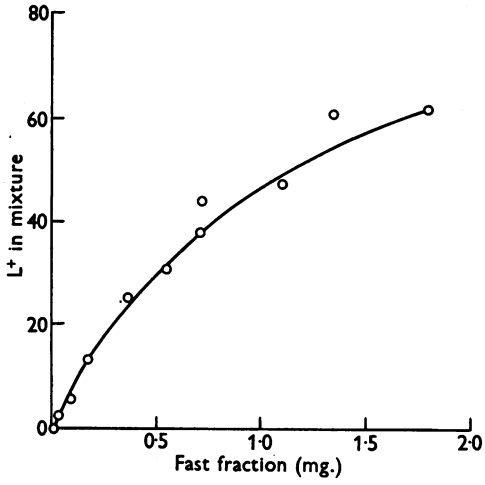


Fig. 9. Effect on the anti-leucocidin-combining power of addition of the fast fraction to a constant amount of the slow fraction. Assay was by the combining-power method. The mixtures contained 0·076 mg. of slow fraction.

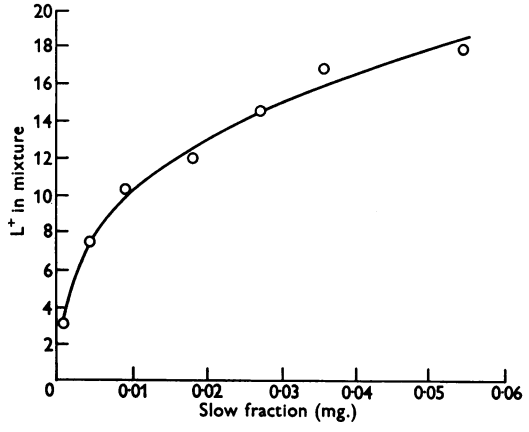


Fig. 10. Effect on the anti-leucocidin-combining power of addition of the slow fraction to a constant amount of the fast fraction. Assay was by the combining-power method. All mixtures contained 0·22 mg. of fast fraction.

The displacement eluate 1 has not been studied in any detail. The precipitate stored in saturated $(NH_4)_2SO_4$ was brown. Boundary electrophoresis in 0·1M-acetate buffer, pH 5·0, showed two peaks in the ascending limb and these were fused in the descending limb in the way characteristic of the hydroxylapatite product of culture filtrate P9 (Fig. 2). Immunological analysis by the Ouchterlony method revealed that in addition to the three bands characteristic of the fast fraction of leucocidin other antigens were present in solutions more concentrated than 0·2%.

### *Properties of mixtures of the fast and slow fractions*

Boundary electrophoresis of mixtures of the fast and slow fractions in 0·2M-phosphate buffer, pH 7·0, showed a single peak, but as the mobilities of the isolated components are similar at this pH value, this cannot be taken as evidence for complex formation.

Figs. 9 and 10 show the effect of varying amounts of each fraction in the presence of a constant

Table 7. *Leucocidin activity of a mixture of the slow and fast fractions*

All mixtures contain 1 $\mu$g. of slow fraction. Assay was by comparison of the activities of twofold dilutions.

| Fast fraction ($\mu$g.) | 0 | 2·0 | 4·8 | 5·8 | 10 | 20 | 40 |
|---|---|---|---|---|---|---|---|
| MLeD in mixture | 20 | 400 | 800 | 1600 | 2400 | 2400 | 2400 |

amount of the other on the leucocidin titre determined by the combining-power method. Table 7 shows the effect of varying amounts of the fast fraction with a constant amount of the slow fraction on the MLeD titre. Ouchterlony plates developed with mixtures of the fast and slow fractions in the antigen wells had the appearance expected from independent development of the bands characteristic of the isolated fractions.

## DISCUSSION

By methods depending upon the molecular charge it has been possible to separate leucocidin into two main fractions. The separation is accompanied by loss of activity, which is regained by mixing the fractions. It has not been possible to prepare either of the two fractions completely free from residual activity but there is evidence that the little activity each has is a consequence of contamination with the other.

The two fractions have a nitrogen content, amphoteric behaviour and ultraviolet absorption consistent with their containing little material other than protein. One of them, the slow fraction, behaves immunologically, electrophoretically and chromatographically as a single substance (except for contamination with the fast fraction). However, it is well known that proteins trail considerably on hydroxylapatite and on carboxymethylcellulose, and it is not considered that either fraction of leucocidin is quite free from trace contamination with other products of the *Staphylococcus*. It is probable that an antiserum prepared against either fraction would contain antibodies to other materials.

The other fraction of leucocidin, apart from the possibility of trace contamination, does not behave as a single substance. Immunological analysis by diffusion in agar shows three bands, of which two persist to small antigen concentrations, and electrophoresis at pH 5·0 shows, in the ascending limb, clear separation of two boundaries. In the descending limb these are less clearly defined and there appears to be a third component separating the regions corresponding to the two major components. This asymmetry can arise in boundary electrophoresis from the partial separation of two components which interact to form a complex. Such interaction between particles of different net charge could account for the failure of the components of the fast fraction to separate on the cation-exchange resins.

It is not known whether all of the components in the fast fraction are necessary, together with the slow fraction, to give leucocidin activity. It is also not known if there is interaction between the slow and fast fractions at pH 7·0. As precipitin bands corresponding to the isolated fractions are produced by mixtures it is clear that the interaction, if it exists, is weak and that some of the free fractions are also present. Moreover, separate antibodies to each are present in staphylococcal antitoxin, suggesting that in the bacterial filtrate the two are not in a complex. It is therefore possible that the death of the white blood cell induced by leucocidin is due to the two components acting completely independently, the action of each, alone, being such that the morphological changes described by Gladstone & van Heyningen (1957) or the inhibition of respiration are not brought about.

Mixtures of the fast and slow fractions have a maximum activity when the ratio of the former to the latter is about 10:1. This figure is subject to the lack of precision of the activity assay. The more precise combining-power method gives an ambiguous result. The shape of the curves in Figs. 9 and 10 does not permit calculation of the ratio of fast to slow at maximum combining power, but if the ratios are compared for (maximum combining power)/2, Fig. 9 gives a value of 9 and Fig. 10 a value of 27·5. This divergence is not unexpected, for the antiserum contains antibodies to both components and the antibody-combining-power method of assay would give a meaningful value for the optimum ratio of the components only if there were a stable complex formed which was responsible for the activity, and if the antiserum contained a specific antibody to this complex. There is no evidence that this is the case. It also follows from these considerations that the antibody-combining-power method of leucocidin assay must be treated with caution. It has been shown that a particular $L^+$ value can result from mixtures of quite different composition. Similarly it is impossible to set up a standard antitoxin unit, for different antisera may contain different proportions of the different antibodies.

When the work described in this paper was started it was assumed that there was a single substance responsible for the leucocidin activity. It is not now considered that the method used to

isolate the two fractions of leucocidin is necessarily the most efficient. Many other fractionation procedures than those described in this paper were tried but gave poor recoveries of the activity. It is now clear that this may have been due to separation of the fractions and not to irreversible inactivation. Nevertheless the method of purification described here permits the production of highly purified materials in reasonable yield and these are suitable for assessing the significance of leucocidin in staphylococcal pathogenicity. The separation of leucocidin into two fractions will permit controlling of such biological experiments; any effect possessed by one component, if it is due to an action on the white blood cell, must be multiplied manyfold by addition of the other.

## SUMMARY

1. A medium suitable for large-scale production of leucocidin in high titre by *Staphylococcus aureus* has been developed.

2. An assay method for leucocidin suitable for routine use, based on the ability of leucocidin to prevent reduction of phenol-indophenol-2:6-dichlorophenol by macrophages has been developed.

3. Crude leucocidin can be partially purified by fractionation with ammonium sulphate and on hydroxylapatite.

4. On columns of cation-exchange resins leucocidin separates into two main fractions. There is loss of activity during the separation which is regained by mixing the fractions.

5. The nitrogen contents, ultraviolet-absorption and amphoteric behaviour of the fractions of leucocidin are characteristic of proteins.

I am greatly indebted to Dr D. W. Henderson of the Microbiological Research Establishment, Porton, for providing the facilities for the large-scale production of the culture filtrates; to Mr R. Elsworth and Mr R. Telling for carrying this out; to Miss Mollie Barr of the Wellcome Research Laboratories for the staphylococcal antitoxin; to Dr W. E. van Heyningen for his valued criticism and advice; and to Miss P. Womar and Mr L. Taylor for their technical assistance.

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# The Starch of the Tuber and Shoots of the Sprouting Potato

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(*Received* 18 *March* 1959)

Relatively few chemical investigations have been carried out on the amylose content of the potato-starch granule during growth (see Greenwood, 1956). Meyer & Heinrich (1942) isolated starch from the leaves, shoots and tubers and reported the amylose contents to be 22, 73 and 37 % respectively. The percentage of amylose determined by potentiometric iodine titration of the starch in the growing tuber was shown by Halsall, Hirst, Jones & Sansome (1948) to remain constant at 17 % for two varieties. These authors also found the average length of unit chain of the amylopectin components (calculated from periodate oxidation results on the whole starch) to be constant at 24–26 anhydroglucose units. There is, however, evidence that the percentage of amylose may vary with the botanical variety of the tuber. Anderson & Greenwood (1955) have found small variations in iodine affinity of