responsible for the appreciable quantity of lysophosphatidylethanolamine found to be present. Although it is possible that some breakdown of phosphatidylethanolamine occurs during the process of evaporating the fractions from the alumina column in preparation for silicic acid chromatography, this seems unlikely to be extensive. It is probable therefore that lysophosphatidylethanolamine is a normal constituent of rat brain, and is not an artifact produced by experimental manipulations.

SUMMARY

1. Chromatography of mixed rat-brain lipids on an alumina column by a gradient method, in which the water content of a chloroform–methanol solvent was raised from 7 to 12·5% (by vol.), resulted in the elution of a peak containing all the ethanolamine-based phospholipids.

2. The main components were phosphatidylethanolamine and ethanolamine plasmalogen, but smaller quantities of sulphatide, lysophosphatidylethanolamine and 'excess ester' were also present.

3. Chromatography of this partially purified material on silicic acid, whereby the methanol content of a chloroform–methanol solvent system was gradually raised from 2 to 9% (by vol.), produced a satisfactory separation into the following fractions: (*a*) 'excess ester'; (*b*) a mixture of phosphatidylethanolamine and ethanolamine plasmalogen; (*c*) a mixture of sulphatide and lysophosphatidylethanolamine.

4. Phosphatidylethanolamine and ethanolamine plasmalogen have been found to be moderately stable when in contact with alumina.

We wish to express our thanks to Dr G. H. Sloane-Stanley for making available his unpublished procedure for aldehyde determination, and to Dr G. C. R. Morris for help with the potassium determinations.

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The Effect of Staphylococcal Leucocidin on the Leucocyte

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Staphylococcal leucocidin consists of two proteins that act synergistically to kill the leucocytes of humans and rabbits (Woodin, 1960). Observations on the cytotoxicity of leucocidin have been restricted to the use of simple viability tests such as the ability of leucocidin to inhibit respiration or produce morphological changes in susceptible cells. The present paper describes some biochemical changes in the leucocyte treated with leucocidin.

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EXPERIMENTAL

Materials

Leucocytes. These were obtained from peritoneal exudates 5–10 hr. after injection of 0·85% NaCl. The exudate, to which heparin (10 mg./1000 ml.) was added, was filtered through surgical gauze and centrifuged at 200*g* for 5 min. The cells were then washed three times by centrifuging at 200*g* for 5 min. in Hanks medium or a Krebs–Ringer solution (see below) and suspended in one of these media, and a sample was taken for counting. The data in this paper, unless otherwise stated, refer to rabbit leucocytes.

Macrophages. These were taken from the peritoneal cavity 4–5 days after the injection of 80 ml. of liquid paraffin. They were then treated in the same way as the leucocytes. The data in this paper, unless otherwise stated, refer to rabbit macrophages.

Suspension media for the leucocytes and macrophages. According to the conditions of the experiment the media used were: Hanks medium (Hanks, 1948); Krebs–Ringer bicarbonate (Umbreit, Burris & Stauffer, 1957); Krebs–Ringer bicarbonate from which phosphate was omitted; Hanks medium containing 0·5% of gelatin or Krebs–Ringer bicarbonate containing 10% of dextran (Dextran 10; Pharmacia, Uppsala, Sweden). Unless otherwise recorded, to all these media glucose (2 mg./ml.) was added.

Leucocidin. The two components of leucocidin were crystallized by the methods of Woodin (1960) and stored at 4° in saturated $(NH_4)_2SO_4$. Before use the suspended solid was dissolved in, and dialysed against, 0·85% NaCl, pH 5–6. Solutions of either component, about 2 mg./ml., stored at 4° have shown no change in activity over several weeks.

Streptolysin O. This was provided by Professor A. Bernheimer and stated to contain about 50 000 haemolytic units/mg. Before use it was dissolved in 0·1 M-cysteine–HCl in Hanks medium, adjusted to pH 7·2.

Adsorption of leucocidin by the leucocyte

Leucocytes were suspended in 0·5% gelatin in Hanks medium and tubes set up containing various amounts of cells. A constant amount [9·6 μg. of *S* ('slow') component, 8·4 μg. of *F* ('fast') component] of one or both of the leucocidin components was added, and the mixtures were made up to 9 ml. with 0·5% of gelatin in Hanks medium and incubated for 10 min. at 37°. The suspensions were centrifuged at 650*g* for 5 min. and the supernatants assayed for the two components of leucocidin by the Neisser & Wechsberg method (Woodin, 1959). When the *F* or the *S* component of leucocidin was added separately the assay could be carried out directly on the supernatant. When both components were present it was necessary to dialyse the supernatant for 6 hr. against 0·85% NaCl to remove reducing material.

Investigation of the leucocidin effects

Incubation conditions. Suspensions of leucocytes, 10^9–10^7/ml., were incubated at 37° for 10 min., usually one sample containing leucocidin and one not. Unless otherwise recorded each component was added to give 2 μg./10^8 cells. Test tubes were usually used and the cells kept evenly suspended by gentle rocking.

Homogenization of cells. After incubation the suspensions were centrifuged for 5 min. at 650*g* in graduated tubes and the supernatant was removed from the cell pellet. The pellets were suspended in about 5 ml. of homogenization medium and disrupted by treatment for 3 min. in the Mullard ultrasonic generator (Measuring and Scientific Equipment Ltd., Crawley, Sussex). The tubes were kept in ice during this treatment. For homogenization in Potter–Elvehjem tubes the cell pellets were mixed with an equal volume of the homogenization medium and homogenized for 10 min., the tubes being cooled in ice. Microscopic examination showed 100% cell breakage under these conditions. The homogenate was then diluted to 10 times the original volume of the cell pellet and centrifuged at 110 000*g* for 45 min. at 0°. In this way three fractions were obtained: the cell supernatant, the soluble fraction of the homogenate and the insoluble fraction of the homogenate.

Nucleic acids of the leucocyte. After incubation of the cells in Hanks medium, the three fractions were prepared as described, homogenization being carried out in Hanks medium with Potter–Elvehjem tubes. The method of Schneider (1945) was used to isolate the nucleic acids. The ribonucleic acid in the extract was estimated by the orcinol reaction (Ceriotti, 1955), and the deoxyribonucleic acid by the diphenylamine reaction (Burton, 1956). The standard in the orcinol reaction was a sample of yeast nucleic acid (British Drug Houses Ltd., Poole, Dorset) which was partially purified by the method of Kunitz (1941) and contained 8·9% of P. The standard in the diphenylamine reaction was a sample of thymus deoxyribonucleic acid, prepared by the method of Kay, Simmons & Dounce (1952), which contained 9·2% of P.

Phosphoprotein of the leucocyte After incubation of the cells in Hanks medium the cell suspensions were cooled in ice and ice-cold trichloroacetic acid was added to give 10% (w/v). The phosphoprotein (determined as alkali-labile P) was estimated by the method of Schmidt & Thannhauser (1945) except that extraction with chloroform–methanol (2:1,v/v) was substituted for extraction with ethanol and ether.

Lipids of the leucocyte. After incubation of the cells in Hanks medium the cell suspensions were centrifuged at 2600*g* for 15 min. and the supernatant was removed. Chloroform–methanol (20 vol.; 2:1, v/v) was added and the suspension left 4–5 hr. at room temperature. The suspension was filtered and the filtrate washed with 2 mM-$CaCl_2$ by the method of Folch, Lees & Sloane-Stanley (1957). The chloroform-soluble fraction was dried in a rotary evaporator and the solids were dissolved in chloroform. For analysis of the lipid constituents samples were dried in a rotary evaporator. For the determination of dry weight the sample was subsequently dried *in vacuo* over P_2O_5. For separation of the phospholipids samples of the chloroform solution containing 50–90 μg.atoms of P were passed down a column containing 10 g. of silicic acid in chloroform (Mallinkrodt A.R.) maintained at 4°. The column was washed with about 100 ml. of chloroform and eluted with chloroform–methanol (9:1, v/v) and then with chloroform–methanol (2:3, v/v). Fractions were collected and the phosphorus content was determined.

Respiratory mechanisms of the leucocyte and macrophage. (*a*) *Glycolysis in whole cells.* Duplicate suspensions of leucocytes or macrophages (10^7/ml.) were incubated in glucose-free Hanks medium for 15 min. at 37°, one suspension containing leucocidin. A sample (0·5 ml.) was taken from each suspension and deproteinized with an equal volume of trichloroacetic acid (10%, w/v). Glucose (2 mg./ml.) and KCN (50 μg./ml.) were then added to the remaining cell suspensions and incubation was continued at 37° for 30 min., when a further sample (0·5 ml.) of each suspension was deproteinized. The lactic acid present in the deproteinized solutions was then determined (Barker & Summerson, 1941).

(*b*) *Glycolysis in homogenates.* Homogenates of normal and leucocidin-treated leucocytes were prepared in Hanks medium in Potter–Elvehjem tubes and diluted with 0·154 M-KCl. Mixtures were then set up containing, in 3 ml.,

150 μg. of KCN, an amount of homogenate corresponding to $0{\cdot}4 \times 10^8$ cells and the cofactors and substrates given by Umbreit *et al.* (1957) as suitable for the measurement of glycolysis in liver homogenates. A third mixture (3 ml.) containing homogenate from leucocidin-treated leucocytes contained also 1 mg. of *F* component of leucocidin and 1 mg. of *S* component of leucocidin. Samples of the mixtures (1 ml.) were deproteinized immediately after adding the homogenate and after incubation for 30 min. at 37°. The lactic acid in the deproteinized solution was determined. Preliminary experiments indicated that lactate production was proportional to the amount of homogenate added when that was less than the equivalent of 10^8 cells.

(*c*) *Oxygen uptake in whole cells.* Leucocytes or macrophages were suspended in Krebs–Ringer phosphate solution and O_2 uptake was measured in Warburg manometers, with 2 ml. suspensions, 10^8 cells/ml. After about 20 min. leucocidin was tipped in from the side arm and measurement continued for a further 60 min.

Water, Na^+ and K^+ ion contents of the leucocyte and macrophage. Cells suspended in Hanks medium (2 ml. containing 10^8 cells) were incubated in weighed tubes and then centrifuged at 650*g* for 4 min. The supernatant was removed, and the tubes were drained, weighed, dried at 110° and weighed again. The dried residue was ashed in HNO_3 and the Na^+ and K^+ ion contents were determined by flame photometry (Amoore, Parsons & Werkheiser, 1958).

Acid-soluble constituents of the leucocyte. After incubation in phosphate-free, glucose-free Krebs–Ringer bicarbonate solution the cell suspensions (12 ml. containing 4×10^9 cells) were cooled in ice and made 0·5M with respect to $HClO_4$. Extraction was continued for 30 min. at 0° and the suspensions were then centrifuged at 80 000*g* for 1 hr. The supernatant was removed and analysed. The $HClO_4$ was removed when necessary, as the potassium salt. For some experiments the cell suspensions, after incubation, were centrifuged at 300*g* for 5 min., and the supernatants and cell residues separately treated with $HClO_4$. In calculating the amount of an acid-soluble constituent the anhydrous volume of material precipitated by $HClO_4$ was neglected. In the estimation of the ninhydrin-reacting material glycine was used as a standard.

For paper chromatography of the ninhydrin-reacting material in the acid extracts of the cell residues and supernatants, these were separated after incubation and extracted with trichloroacetic acid (final concn. 10%, w/v) in the same way as for the $HClO_4$ extraction. The trichloroacetic acid was then extracted into ether and the water-soluble fraction passed down a column of Zeo-Karb 225 in the H^+ form (Permutit Co. Ltd., London) with about 5 ml. of resin for 10 ml. of extract. The columns were washed free from Cl^- ions and then eluted with 0·5N-NH_3 solution. The eluate before and for 25 ml. after the appearance of free NH_3 in the eluate was then freeze-dried. The solid was dissolved in a small volume of water and applied to Whatman no. 1 paper, and a chromatogram developed with butanol–acetic acid–water (60:10:40, by vol.). The paper was sprayed with ninhydrin in butanol.

Non-diffusible material liberated from leucocytes by leucocidin. After incubation of the cells in Hanks medium (30 ml. containing 10^{10} cells) a sample (10 ml.) of the supernatant from leucocidin-treated cells was isolated and dialysed for several days against distilled water, and the suspension of precipitated material dried at 110° in a weighed ampoule. The N content of the dry solid was then determined. Further samples (2 ml.) of the leucocidin-treated leucocyte supernatant were analysed for P, cholesterol, fatty acid and esterified fatty acid. For electrophoresis and ultracentrifuging a sample (5 ml.) of the supernatant of leucocidin-treated cells was dialysed against 0·1M-NaCl–0·02M-tris buffer, pH 7·5.

Distribution of protein in the leucocyte and macrophage. After incubation of the cells in Hanks medium (30 ml. containing 10^{10} cells) the cell supernatant and the soluble fraction and insoluble fraction of the homogenate were prepared. The three fractions were precipitated and washed with 10% (w/v) trichloroacetic acid. The extraction with trichloroacetic acid was followed by extraction with ethanol (10 ml.), and it was apparent that much protein dissolved in the ethanol. After extraction with ethanol the nucleic acids were dissolved by heating for 15 min. in 5% (w/v) trichloroacetic acid (10 ml.) at 90°. The N contents of the material soluble in ethanol and the material insoluble in ethanol and insoluble in hot trichloroacetic acid were then determined. The protein contents were calculated from the sum of these values, assuming these proteins to contain 16% of N. The N contents of the fractions will contain a contribution from the N-containing lipids. This is very small (see below) and was the same for both normal and leucocidin-treated leucocytes.

To prepare solutions for analytical ultracentrifuging, the cell pellets of 10^{10} normal and 10^{10} leucocidin-treated leucocytes were each homogenized in 0·3M-sucrose in Potter–Elvehjem tubes. The homogenates were diluted to 10 ml. with 0·3M-sucrose and the insoluble fraction of the homogenate was centrifuged down. The supernatants were then dialysed against 0·1M-NaCl–0·02M-tris buffer, pH 7·5.

Lysozyme and phagocytin of the leucocyte. After incubation the leucotye suspensions (5 ml. containing $2 + 10^9$ cells) were centrifuged at 650*g* for 5 min. and the supernatants separated. The cell pellets were then homogenized and the sucrose-soluble and citric acid-soluble fractions prepared by the method of Hirsch (1960). The lysozyme and phagocytin contents were then determined by the methods used by Hirsch (1960). Lysozyme contents are given as unit weights of egg-white lysozyme. Phagocytin units are the dilutions at the end points.

Readsorption of the proteins present in the leucocidin-treated leucocyte supernatant. After incubation of leucocytes in Hanks medium the cell supernatants were isolated. The insoluble fractions of normal and leucocidin-treated leucocytes homogenized ultrasonically in Hanks medium were then prepared and washed three times in Hanks medium by centrifuging at 36 000*g* for 30 min. at 0°. They were then diluted in Hanks medium to the original volume of the cell suspensions. Tubes were then set up with constant volumes (containing 3·5 mg. of protein) of the leucocidin-treated cell supernatant and various volumes of the washed insoluble residues of the homogenized cells. After mixing, the tubes were left at room temperature for 10 min. and centrifuged at 36 000*g* for 30 min. at 0°. The trichloroacetic acid-insoluble N of the supernatants was then determined.

Analytical methods

Chemical methods. These were: reducing sugar, Park & Johnson (1949); amino N, Yemm & Cocking (1955); lipid-bound amino N, Lea & Rhodes (1954); esterified fatty

acid, Morgan & Kingsbury (1959); fatty acid, Dole (1956); cholesterol, Henly (1957); free and total aldehyde, Gray & Macfarlane (1958); hexose diphosphate, hexose monophosphate, adenosine diphosphate and adenosine triphosphate, Slater (1953); inorganic P, Berenblum & Chain (1938). For the analysis of the acid extracts of leucocytes the inorganic P was precipitated as the calcium salt (Delory, 1938). The inorganic P was also determined, without precipitation as the calcium salt, but after incubating the extract of the leucocyte for 30 min. at room temperature in the acid–molybdate reagent of the Berenblum & Chain (1938) method. The difference between the values for the inorganic P determined by calcium precipitation and that found after incubation in the acid–molybdate reagent was taken as the creatinine phosphate content. Acid-labile P was determined as the inorganic P liberated after hydrolysis at 100° in N-H_2SO_4 for 10 min. For determination of organically-bound P samples were digested in conc. H_2SO_4–60% (w/v) $HClO_4$ (3:2, v/v). N was determined by micro-Kjeldahl.

Analytical ultracentrifuging. This was done in a Spinco model E ultracentrifuge.

Electrophoresis. This was done in an Antweiler electrophoresis apparatus.

RESULTS

Adsorption of the two components of leucocidin by the leucocyte. Fig. 1 shows that each component of leucocidin is adsorbed by rabbit leucocytes. When both components are present there is greater adsorption of both, indicating that there is no competition for the sites at which adsorption occurs. When saturated with each component leucocytes contain 1 μg./10^8 cells, corresponding to 10^5 molecules/cell. No adsorption on guinea-pig leucocytes could be detected. The insensitivity of guinea-pig leucocytes to the lethal effects of leucocidin has been noted by Gladstone & van Heyningen (1957).

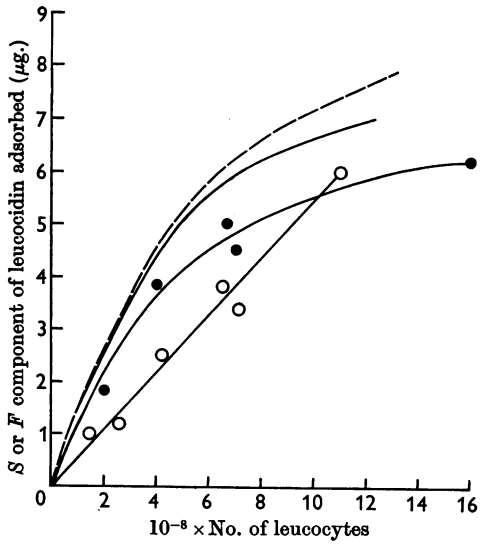

Fig. 1. Adsorption of leucocidin by rabbit leucocytes. ○, *F* component; ●, *S* component; – – –, *S* component in the presence of the *F* component; —, *F* component in the presence of the *S* component. The experimentally determined points for the adsorption of each component in the presence of the other have been omitted. The amounts of the *F* and *S* components added were 8·4 μg. and 9·6 μg. respectively in a total volume of 9 ml.

Tests for enzymic activity in the leucocidin components. Solutions containing the *F* or the *S* component of leucocidin (1 mg./ml.) and a solution containing both components at this concentration were found to be free from the following activities: ribonuclease (Kunitz, 1941), deoxyribonuclease (Laskowski & Seidel, 1945), staphylokinase (Bungay, 1961), staphylococcal haemolysin (Glenny & Stevens, 1935), egg-yolk turbidity-producing factor (Gillespie & Adler, 1952). On incubation with casein (2·0% in 0·85% NaCl, pH 7·5), no inorganic P was liberated. On incubation with ribonuclease (0·5% in Hanks medium) there was no increase in amino N. Neither component hydrolysed diphosphopyridine nucleotide, reduced diphosphopyridine nucleotide or triphosphopyridine nucleotide (Dr R. van Heyningen, unpublished results). It is known that many of these activities are present in some staphylococcal filtrates (Elek, 1959), and their absence from leucocidin is evidence for the homogeneity of the two components.

Effect of leucocidin on some structural components of the leucocyte. Only the insoluble fraction of homogenized leucocytes contained nucleic acid, and the values (ribonucleic acid, 0·15 mg./10^8 cells and deoxyribonucleic acid, 0·6 mg./10^8 cells) were identical for both normal and leucocidin-treated cells. Thus no degradation of nucleic acid accompanies leucocidin action. Similarly, the phosphoprotein-P content (0·08 μmole/10^8 cells) was the same in normal and leucocidin-treated cells. Table 1

Table 1. *Effect of leucocidin on the content of some acid-soluble constituents of the leucocyte*

After incubation in glucose-free, phosphate-free Krebs–Ringer solution the leucocyte suspensions were treated with $HClO_4$ before analysis.

Material	Amount in extract of 10^8 leucocytes: Normal	Leucocidin-treated
Reducing sugar (μg. of glucose)	44	46
Nitrogen (μg.)	34	39
Ultraviolet-absorbing material in 0·5 N-$HClO_4$ (vol. × $E_{260}^{1\,cm.}$)	4·0	4·2
Orcinol-reacting material (μg. of ribonucleic acid)	95	106
Phosphorus (μg.)	34	33
Ninhydrin-reacting material (μg. of glycine)	110	110

gives the concentration of some of the acid-soluble constituents of suspensions of normal and leucocidin-treated leucocytes and, as no significant differences exist between the two sets of values, provides more evidence for absence of degradation of the insoluble structures of the cell by leucocidin.

To detect a change in the lipids of the leucocyte the gross composition of the lipid fraction and the chromatographic behaviour of the phospholipids were investigated. Liberation of water-soluble constituents from the lipids should have been followed by their removal in the cell supernatant or the upper phase produced in washing the chloroform–methanol extract. Changes other than the liberation of water-soluble components should have been reflected in changes in the gross composition or chromatographic behaviour. The chloroform solution derived from the chloroform–methanol extract from 10^8 leucocytes contained 1·38 mg. of solids, 0·96 μmole of P, 1·8 μequiv. of esterified fatty acid, 0·14 μequiv. of total aldehyde, 0·002 μequiv. of free aldehyde, 0·31 μequiv. of amino N, 0·8 μmole of total N, 0·47 μmole of cholesterol. No differences could be detected in the composition of extracts of normal and leucocidin-treated leucocytes. Fig. 2 shows the separation curves for the phospholipids of normal leucocytes chromatographed on silicic acid, and five main bands can be distinguished. These were bulked as shown in Fig. 2. Between bands *A* and *B*, bands *C* and *D* and bands *D* and *E* respectively, the P content of the effluent did not fall to zero and the points of separation of the bands were taken as the fraction with the lowest P content. Plasmalogen and amino N were found in band *B* only; choline was present in bands *D* and *E* only; Band *B* is clearly heterogeneous, but as the degree of overlap varied in different experiments no assessment could be made of the relative proportions of the constituents. Except for variations that could be ascribed to inadequate separation of the constituents of band *B*, no differences could be detected in the chromatographic behaviour of extracts from normal and leucocidin-treated leucocytes. In an experiment, in which extracts of normal and leucocidin-treated leucocytes were chromatographed on columns prepared identically, bands *A*–*E* contained 4·5, 28, 9, 28 and 24% respectively of the phospholipid P from normal leucocytes, and 3·7, 31, 11, 25 and 23% respectively of the phospholipid P of leucocidin-treated leucocytes.

Effect of leucocidin on the respiratory mechanisms of the leucocyte and macrophage. The inhibition of the ability of leucocytes to reduce certain respiratory dyes is the basis of the Neisser & Wechsberg (1900) method for detecting leucocidin activity. Consequently the possibility that leucocidin exerted a direct effect on the respiratory mechanisms of the cell was investigated. Table 2 shows that after treatment with leucocidin there is a decrease in the amount of lactic acid produced in whole leucocytes and macrophages but that there is no effect of leucocidin on lactate production in homogenates of the leucocyte.

Lactate production by whole rabbit leucocytes or macrophages was not affected by addition of only one of the two components of leucocidin, and lactate production by whole guinea-pig macrophages was not affected by the addition of both components of leucocidin.

It is known that macrophages can live for several days under anaerobic conditions (Harris & Barclay, 1955) and, similarly, glycolysis alone can provide the source of energy for many of the vital functions of the leucocyte (Elsbach & Schwartz, 1959; Sbarra & Karnovsky, 1959). It was therefore improbable that leucocidin exerted its toxic effect through inhibition of O_2 uptake, and in both leuco-

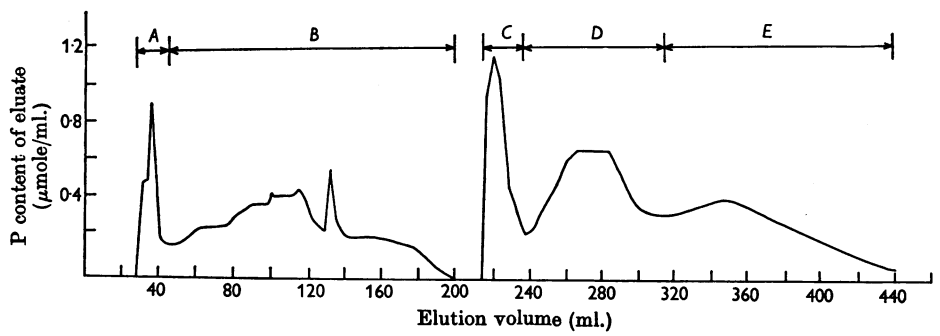

Fig. 2. Fractionation of the phospholipids of the leucocyte. An amount of lipid containing 75 μmoles of P was added to a 10 g. column of silicic acid. Fractions (2·2 ml.) were collected in the elution range 0–240 ml. and 5·5 ml. fractions in the elution range 240–500 ml. The elution volumes are measured after changing the eluent from chloroform to chloroform–methanol (9:1, v/v). At 200 ml. of elution volume, the eluent was changed to chloroform–methanol (2:3, v/v). *A*, *B*, *C*, *D* and *E* are bulked fractions.

cytes and macrophages there was no decrease in O_2 uptake till 15–20 min. after addition of leucocidin.

Permeability changes induced by leucocidin in leucocytes and macrophages. Table 3 shows that on incubation with leucocidin the cell swells, loses K^+ and gains Na^+ ions. No differences in water, K^+ or Na^+ ion content have been found in cells incubated with leucocidin for 5–20 min. These observations were made on centrifuged cell pellets. Elsbach & Schwartz (1959) showed that in the presence of fluoride and KCN leucocytes swell to the same extent as that found here for leucocidin-treated leucocytes. They measured the extracellular volume in centrifuged cell pellets similar to those prepared here and obtained values of 18% for normal cells and 40% for swollen cells. (The increased extracellular volume of the swollen cells probably results from their decreased density and less close packing during centrifuging.) Calculations based on these values give the K^+ ion concentration in μequiv./ml. of cell water to be 102 in normal cells and 19 in leucocidin-treated cells and establish that the changes in the K^+ ion content of the cell pellets cannot result from a changed extracellular volume. No changes in water or electrolyte content were observed when leucocytes or macrophages were incubated with either the *F* or the *S* component of leucocidin alone. The two components of leucocidin mixed had no effect on the water and electrolyte content of guinea-pig macrophages.

Table 4 shows that while about 75% of the K^+ ions of the cell is lost by leucocidin-treated leucocytes there is only a slight loss of amino acids (estimated as ninhydrin-reacting material), nucleotides (estimated as acid-soluble, ultraviolet-absorbing material) or P. No qualitative differences could be detected by paper chromatography of the

Table 2. *Effect of leucocidin on lactate production by leucocytes and macrophages*

Cells were incubated, alone or with added leucocidin, for 15 min. in glucose-free Hanks medium. For glycolysis in whole cells glucose and KCN were then added and incubation was continued for 30 min. For glycolysis in homogenates, cells were homogenized after the preliminary incubation and added to a mixture of substrates and cofactors, and incubation was continued for 30 min.

Glycolysis system	Lactate produced (μmoles/hr./10^8 cells)
Normal leucocytes	4·4
Leucocidin-treated leucocytes	1·1
Homogenate from normal leucocytes	6·3
Homogenate from leucocidin-treated leucocytes	6·2
Homogenate from leucocidin-treated leucocytes with added leucocidin	6·8
Normal macrophages	5·7
Leucocidin-treated macrophages	2·6

Table 3. *Effect of leucocidin on the water and electrolyte content of the leucocyte and macrophage*

The leucocytes were suspended in Krebs–Ringer phosphate solution and the macrophages in Hanks medium. The cells were incubated for 5 min., alone or with added leucocidin, and centrifuged, and the cell pellets analysed.

Cell	Water content (% of wet wt.)	K^+ ion content (μequiv./ml.)	K^+ ion content (μequiv./g.)	Na^+ ion content (μequiv./ml.)	Na^+ ion content (μequiv./g.)
Normal leucocyte	81	85	435	112	575
Leucocidin-treated leucocyte	92	14	155	175	1950
Normal macrophage	80	74	350	110	510
Leucocidin-treated macrophage	88	18	210	160	1800

Table 4. *Retention of some acid-soluble compounds by leucocidin-treated leucocytes*

Data are calculated from a series of similar experiments. The retention in the leucocidin-treated cell (column 2) is the amount in the pellet of leucocidin-treated cells expressed as percentage of the amount in the pellet from the normal cells. The distribution between the supernatant and leucocidin-treated cell (column 3) is the ratio concn. in pellet from leucocidin-treated cells/concn. in supernatant from leucocidin-treated cells.

Material	Retention in leucocidin-treated cell	Distribution between supernatant and leucocidin-treated cell
Potassium	25	1·65
Ninhydrin-reacting material	82	10·0
Ultraviolet adsorption at 260 mμ in N-HCl	85	16·5
Total nitrogen	80	13·0
Orcinol-reacting material	78	30·0
Phosphorus	68	6·5
Hexose monophosphate	95	—
Hexose diphosphate	100	—

Table 5. *Effect of leucocidin on the distribution of some phosphorus compounds in leucocyte suspensions*

After incubation in phosphate-free Krebs–Ringer bicarbonate the cell supernatants and centrifuged pellets were cooled in ice and treated with ice-cold trichloroacetic acid.

Cell fraction	Phosphorus content (μmole/10^8 cells)			
	Inorganic P	Creatine phosphate	Acid-labile phosphate	Total organic P
Normal-cell supernatant	0·028	0	0	0
Leucocidin-treated-cell supernatant	0·25	0·01	0·02	0·05
Normal-cell pellet	0·062	0·076	0·25	0·81
Leucocidin-treated-cell pellet	0·165	0·085	0·05	0·405

ninhydrin-reacting material in the supernatants or cell pellets of normal and leucocidin-treated leucocytes. In contrast 80 % of the P found in the supernatant of leucocidin-treated leucocytes is inorganic and thus differs from the P retained in the cell pellet.

Accumulation of inorganic phosphorus in the leucocidin-treated leucocyte. Table 1 shows that there is no net increase in the acid-soluble P of the leucocyte treated with leucocidin. Also, there is a release of inorganic but only little organically-bound P into the supernatant of leucocidin-treated leucocytes (Table 5). The analysis of the effect of leucocidin on the composition of the acid-soluble P compounds of the leucocyte was therefore extended and it was found that in leucocyte suspensions treated with leucocidin there is an accumulation of inorganic P (0·3–0·37 μmole/10^8 cells) and a corresponding decrease in the acid-soluble organically-bound P. The inorganic P and creatinine phosphate contents of normal leucocyte suspensions in phosphate-free Krebs–Ringer bicarbonate did not change during incubation for 30 min. at 37°.

The accumulation of inorganic P in suspensions of leucocidin-treated leucocytes was not prevented by addition of NaF (final concn. 0·2 M) or NaF and KCN (final concn. 0·2 M and 2 mM respectively) before addition of leucocidin. These substances did not prevent the liberation of the usual amount of protein into the supernatant of leucocidin-treated leucocytes (see below). The addition of adenosine triphosphate (4 mg./ml.) to the leucocytes subsequently incubated with leucocidin did not increase the accumulation of inorganic P. Leucocytes treated with streptolysin O, or disintegrated ultrasonically and then incubated in Hanks medium for 10 min., also liberated a similar amount of inorganic P, but in both these cases the effect was abolished by addition of fluoride. There was no accumulation of inorganic P in leucocytes incubated with iodoacetate (1 mM) or iodoacetate and cyanide (1 mM and 2 mM respectively).

Table 6 shows an analysis of some of the organic P compounds of the leucocyte and shows that there is a decrease in the adenosine triphosphate content of the leucocyte after leucocidin action. Hydrolysis to adenosine monophosphate could account for the accumulation of 50–60 % of the inorganic P. Hydrolysis to adenosine would account for nearly all the inorganic P accumulation.

Table 6. *Effect of leucocidin on the composition of the acid-soluble phosphorus compounds of the leucocyte*

	μmole/10^8 cells present in	
	Normal cells	Leucocidin-treated cells
Hexose diphosphate	0·010	0·010
Hexose monophosphate	0·005	0·005
Adenosine diphosphate	0·010	0·050
Adenosine triphosphate	0·100	0·009
Acid-labile P	0·24	0·095

Liberation of protein from the leucocyte treated with leucocidin. There was a considerable quantity of non-diffusible material in the supernatant of the leucocidin-treated leucocyte. The N content of this material was 14·5 %, indicating that little carbohydrate was present; the P content (0·014 %) indicated absence of significant amounts of phospholipid or nucleic acid, and no cholesterol, esterified fatty acid or unesterified fatty acid could be detected. On electrophoresis at pH 7·5 five components could be distinguished.

Table 7 shows that the release of protein from the leucocyte is not paralleled by a decrease in the soluble protein content of the soluble fraction of the homogenate. Similar results were obtained whether the homogenization was carried out ultrasonically or in a Potter–Elvehjem homogenizer and whether the homogenization medium was Hanks medium or 0·3 M-sucrose. Table 7 also shows that only a small amount of protein is found in the supernatant of leucocidin-treated macrophages. The same amount of protein was liberated from leucocytes treated with leucocidin in the presence of dextran (10 %, w/v), which prevented the cell swelling. Protein was also liberated from leucocytes treated with streptolysin O, but in this case there was a corresponding decrease in the protein content of the soluble fraction of the homogenate.

Fig. 3 shows that the soluble fraction of the homogenate from normal and leucocidin-treated

Table 7. *Effect of leucocidin on the protein distribution of leucocytes and macrophages*

After incubation of the cells alone or with added leucocidin, in Hanks medium, the supernatants were removed and the centrifuged cells homogenized in Hanks medium. The soluble fraction is the supernatant from centrifuging of the homogenate at 110 000*g* for 45 min.

Cell	Protein content (mg./10^8 cells)		
	Cell supernatant	Soluble fraction, homogenized cells	Insoluble fraction, homogenized cells
Normal leucocytes	0·15	1·95	4·7
Leucocidin-treated leucocytes	1·5	1·85	3·5
Normal macrophages	0·03	3·0	—
Leucocidin-treated macrophages	0·3	2·9	—

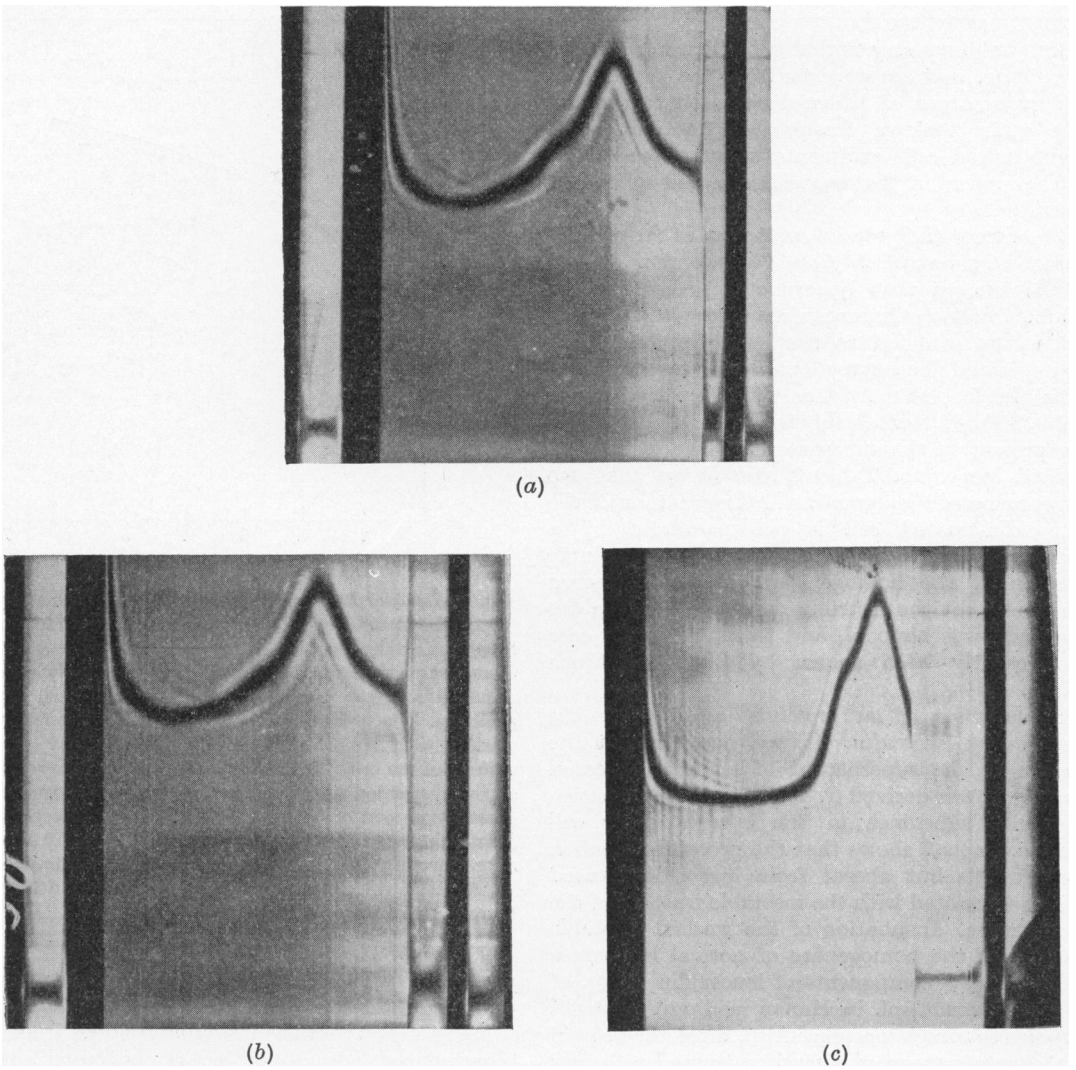

Fig. 3. Ultracentrifuging of the soluble protein derived from normal and leucocidin-treated leucocytes. (*a*) Soluble fraction of homogenized normal leucocytes, 60 min. after reaching 59 780 rev./min. (*b*) Soluble fraction of homogenized leucocidin-treated leucocytes, 53 min. after reaching 59 780 rev./min. (*c*) Supernatant of leucocidin-treated leucocytes, 47 min. after reaching 59 780 rev./min.

Table 8. *Distribution of some antibacterial substances in fractions derived from normal and leucocidin-treated leucocytes*

Lysozyme contents are from comparison of activity with dilutions of egg-white lysozyme. Phagocytin units are the dilutions at the end points.

Cell fraction	Lysozyme content (μg./10^8 cells)		Phagocytin (units/10^8 cells)	
	Normal cell	Leucocidin-treated cell	Normal cell	Leucocidin-treated cell
Cell supernatant	1·0	125	<2	150
Sucrose-soluble fraction of homogenate	110	110	<2	<2
Citric acid extract of sucrose-insoluble fraction	20	20	300	200

leucocytes have very similar behaviour in the ultracentrifuge, and are distinct from the proteins in the supernatant of leucocidin-treated leucocytes. The main peak of the soluble fraction of the homogenized cells sediments about twice as fast as the protein in the leucocidin-treated cell supernatant.

Presence of antibacterial substances in the proteins liberated by leucocidin from the leucocyte. Hirsch (1960) showed that lysozyme is present in the soluble fraction of homogenized leucocytes. Phagocytin was confined to the insoluble fraction of homogenized leucocytes but could be extracted from the insoluble fraction by dilute citric acid. Table 8 shows that both lysozyme and phagocytin are present in the supernatant of the leucocidin-treated leucocyte. Table 8 also shows that the appearance of lysozyme in the supernatant of the leucocidin-treated cell is not paralleled by a decrease in the lysozyme content of the soluble fraction of the homogenized cell. It appeared therefore that the distribution of lysozyme in the leucocyte was bimodal, and that this is the case has recently been shown by Cohn & Hirsch (1960).

Readsorption of the proteins liberated from the leucocyte by leucocidin. It was possible that the protein in the supernatant of leucocidin-treated leucocytes was derived from a hypothetical macromolecular substance in the cytoplasm or cell surface. Table 7 shows that the protein present in normal cells but absent from leucocidin-treated cells is associated with the insoluble fraction of the homogenate. Incubation of the washed insoluble fraction of the homogenate of normal leucocytes with the two components of leucocidin (1 mg. of each component/ml. in Hanks medium) does not give rise to soluble protein. Also, when the protein in the supernatant of leucocidin-treated cells was added to the insoluble fraction of the homogenate it was readsorbed. Thus if leucocidin-treated leucocytes are homogenized in the presence of the cell supernatant then the total amount of soluble protein found in the homogenate is identical with that from homogenized normal cells.

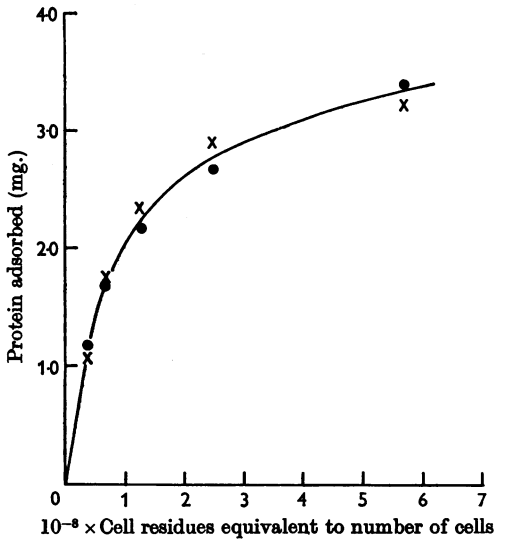

Fig. 4. Readsorption of the protein in the supernatant of leucocidin-treated leucocytes by the insoluble fraction of homogenized leucocytes. After incubation of leucocytes, alone or with leucocidin, the cell supernatants were removed and the centrifuged cells disintegrated ultrasonically. The insoluble fraction of the homogenate was washed with Hanks medium and then diluted to the volume of the original cell suspension. To various volumes of the suspended insoluble fraction of the homogenate a constant amount of the leucocidin-treated cell supernatant (containing 3·5 mg. of protein) was added. After 10 min. at room temperature the soluble protein was determined. ×, Adsorption by residues of normal cells; ●, adsorption by residues of leucocidin-treated cells.

Fig. 4 shows that the insoluble residues from 10^8 homogenized normal, or leucocidin-treated, cells readsorb the same amount of protein, about 2 mg. This is 30% greater than the amount released from normal leucocytes by leucocidin (1·5 mg./10^8 cells, Table 7).

DISCUSSION

It has been found that 10^5 molecules of each component of leucocidin are adsorbed by a leucocyte. The conditions of the adsorption, removal of 80% of the leucocidin from 9 ml. of solution by cells occupying about 2 ml., are such that the adsorption is not to be explained by pinocytosis (Lewis, 1931) but must result from adsorption on the cell surface.

The leucocidin-treated leucocyte rapidly loses most of its potassium but only small amounts of amino acids, nucleotides and phosphate esters. The slightly higher concentration of potassium in the leucocidin-treated cell pellet than that in the supernatant can result from about 5% of the cells being insensitive to leucocidin. The small amounts of amino acids and nucleotides that are released from the leucocyte by leucocidin may result not from increased permeability but from mechanical lysis of a small percentage of the leucocidin-treated leucocytes during the manipulations and centrifuging.

The release of potassium from the leucocidin-treated leucocyte could result from an increased permeability or from an interference with active transport. If the latter were the case then the loss of potassium in the leucocidin-treated leucocyte would be a measure of the rate at which potassium leaks from the normal cell and, as the potassium concentration in the normal cell is constant, of the rate at which it is normally accumulated. The minimum rate at which potassium is lost from the leucocidin-treated leucocyte is 960 μequiv./hr./ml. of cell water. Elsbach & Schwartz (1959) have measured the rate at which potassium is reaccumulated in leucocytes incubated at 37° after exposure to 0°, and from Fig. 2 of their paper it can be calculated to be about 20 μequiv./hr./ml. of cell water. It is therefore improbable that leucocidin acts by interference with the mechanism of active transport.

A change in permeability implies a structural change to the cell surface. This could conceivably be produced by mere combination of the two components with the cell surface. However, no permeability change accompanies adsorption of only one component of leucocidin, and the probability that the two components have an enzymic nature is made more plausible by the following considerations. There is an accumulation of inorganic phosphorus and a decrease in adenosine triphosphate in the leucocidin-treated leucocyte. Because of the failure of fluoride to inhibit this accumulation of inorganic phosphorus it is unlikely that it results from phosphatase action after cellular disorganization. That the partial respiratory failure in the leucocidin-treated leucocyte is not responsible for the accumulation of the inorganic phosphorus is shown by the failure of leucocytes poisoned with iodoacetate or cyanide to show a similar effect. It is possible therefore that the two components of leucocidin, adsorbed on the cell surface, set up a chain of reactions one stage of which requires adenosine triphosphate.

This paper has shown that the soluble protein appearing in the supernatant of the leucocidin-treated leucocyte is not derived from the soluble protein of the cell. It is unlikely that it is derived from the cell surface, for the quantity of protein involved is large and yet the change in permeability of the cell surface is very small. There is indirect evidence that the protein released from the leucocyte by leucocidin is derived from the granules. Gladstone & van Heyningen (1957) have shown that the granules of the leucocyte treated with leucocidin disappear in the first few minutes. Cohn & Hirsch (1960) have recently isolated the granules of the leucocyte and showed that they contain protein and phospholipid but only traces of nucleic acid. Lysozyme and phagocytin activities were found in the protein fraction. These two activities have been found in the proteins released from the leucocyte by leucocidin. Consistent with these proteins' having a granular origin is the fact that little protein is released from macrophages, which lack the characteristic granules of the leucocyte. Hirsch & Cohn (1960) showed that leucocytes lose their granules under a variety of conditions but that there is not a corresponding release of protein into the cell supernatant. As the leucocidin-treated leucocyte retains much of its normal impermeability to small molecules, the release of protein from the leucocyte suggests that the reactions set up by leucocidin have analogies with those involved in the secretion of protein.

SUMMARY

1. The *S* component and the *F* component of leucocidin are both adsorbed by the leucocyte from solutions containing either or both components.

2. Treatment of the leucocyte with leucocidin does not decrease the amount of nucleic acid sedimented by high-speed centrifuging of homogenized leucocytes.

3. The lipids of the leucocyte that are extractable with chloroform–methanol have been analysed and the phospholipids partially separated on silicic acid. The composition and chromatographic behaviour of the lipids is not changed by pretreatment of the cells with leucocidin.

4. Treatment with leucocidin decreases the rate of glycolysis in leucocytes and macrophages but leucocidin has no action on glycolysis in homogenates.

5. After treatment with leucocidin, leucocytes and macrophages swell, lose potassium and gain sodium. The permeability to amino acids, nucleotides and phosphate esters is only slightly changed. There is a release of inorganic phosphorus from the leucocidin-treated leucocyte.

6. There is an accumulation of inorganic phosphorus in the leucocidin-treated leucocyte and a corresponding decrease in the acid-soluble organic phosphorus content. There is a decrease in the adenosine triphosphate content. These effects are not inhibited by fluoride.

7. Protein is released from the leucocidin-treated leucocyte but is not derived from the soluble protein of the cell. Lysozyme and phagocytin activities have been found in the protein released by leucocidin.

8. The protein released by leucocidin from the leucocyte can be readsorbed by the insoluble fraction of homogenized leucocytes.

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The Metabolism of Exogenous *N*-Acetyl-D-glucosamine 6-*O*[^{35}S]-Sulphate in the Normal Rat

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Several workers have observed the excretion of inorganic and ester [^{35}S]sulphate after the administration of chemically synthesized polysaccharide [^{35}S]sulphates to laboratory animals (Morrow *et al.* 1952; Huseman, Hoffman, Lotterle & Widersheim, 1952; Ricketts, Walton & Saddington, 1954). Similar observations were made after the administration of ^{35}S-labelled biosynthetic preparations of chondroitin sulphate to rat (Dohlman, 1956; Dziewiatkowski, 1956) and of heparin to dog (Danishefsky & Eiber, 1959). None of the polysaccharide sulphates used so far is