7. Treatment with leucocidin reduces the amount of [^{14}C]adenine incorporated into adenosine triphosphate and the amount of [^{14}C]acetate incorporated into the lipids of the cell.

8. It is suggested that the stimulation of incorporation of ^{32}P in the leucocidin-treated leucocyte does not result from an increased rate of turnover of any phosphorus compound of the cell but can result from direct utilization at the cell surface of the external orthophosphate.

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The Accumulation of Calcium by the Polymorphonuclear Leucocyte Treated with Staphylococcal Leucocidin and its Significance in the Extrusion of Protein

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This paper shows that there is an accumulation of calcium in the leucocidin-treated leucocyte and that omission of calcium from the medium inhibits the extrusion of the contents of the granules to the exterior of the cell.

METHODS

Materials. Leucocidin and polymorphonuclear leucocytes (referred to in this paper as leucocytes) were obtained and the media used for suspending the cells were prepared as described by Woodin & Wieneke (1963).

Albumin labelled with ^{131}I was obtained from The Radiochemical Centre, Amersham, Bucks., and was diluted with 0·5% human serum albumin in 0·9% NaCl. Human serum albumin was obtained from The Lister Institute, London. The origin of the nucleoside derivatives and the anti-leucocidin antibody is given by Woodin & Wieneke (1963).

Determination of the calcium and magnesium of the leucocyte. Weighed tubes containing 1–5 $\times 10^8$ leucocytes in 5 ml. of ascitic fluid or Hanks medium were incubated for 10 min. at 37°, alone or with added leucocidin and then centrifuged at 3000 rev./min. for 5 min. The supernatants were poured off and the insides of the tubes dried with filter paper. The tubes were weighed, dried at 110° for 18 hr. and weighed again. The dry pellets were heated with 0·5 ml. of conc. HNO_3 at 100° for several hours and then boiled to dryness. The calcium and magnesium contents of the ash were determined.

In a similar experiment two suspensions of 3×10^7 leucocytes in 5 ml. of calcium-free Hanks solution were incubated with leucocidin at 37° for 10 min. in weighed tubes. One suspension was then cooled to 0° and 0·2 ml. of 44 mM-$CaCl_2$ was added to each suspension. Incubation was continued for a further 10 min., one suspension being at 37° and one suspension being at 0°. The suspensions were then centrifuged at 3000 rev./min. and the wet weight, dry weight and calcium content of the cell pellets determined.

Extracellular volume of the centrifuged cell pellets of leucocytes. This was determined on samples of cells incubated at the same time as those used for the determination of the calcium and magnesium contents. After incubation of the cell suspension in weighed tubes, 0·5 ml. of [^{131}I]-albumin (60 000 counts/min.) was added to each tube and the suspension centrifuged immediately. The supernatant was collected and the tubes were weighed, dried at 110° and weighed again. The dry cell residue was dissolved in 0·2 ml. of N-NaOH, diluted with 1·8 ml. of water and samples (0·1 ml.) were taken for determination of the radioactivity. Further samples (0·05 ml.) of the cell supernatant were taken for the determination of radioactivity.

Adsorption of calcium and magnesium by disintegrated leucocytes. Suspensions of 10^9 leucocytes in 5 ml. of Hanks medium were incubated alone or with added leucocidin at

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37° for 10 min. and centrifuged. The supernatants were rejected and the cell pellets homogenized at 0° for 15 min. in Potter–Elvehjem homogenizer tubes. This gave 100% cell breakage. The homogenates were transferred as completely as possible to dialysis sacs, 3 cm. × 0·9 cm. (flat width), which were tied to put the contents under considerable pressure. The sacs were dialysed with stirring for 24 hr. against 1500 ml. of Hanks medium. The contents of the sacs were then transferred to weighed tubes and the wet weights determined. The tubes were dried at 110° for 18 hr. and weighed again. The dry material was ashed and the calcium and magnesium contents were determined.

Preparation of leucocytes in calcium-free media. The cells were washed four times with calcium-free Hanks solution, ten times the volume of the centrifuged cell pellet being used. They were then suspended in calcium-free Hanks medium, or calcium-free, phosphate-free Krebs–Ringer bicarbonate solution, and treated as described below. The washing procedure took 20 min.

Release of protein from the leucocidin-treated leucocyte in calcium-free media. Eight suspensions of 2×10^8 leucocytes in 6 ml. of calcium-free Hanks medium were incubated with leucocidin at 37° for 3 min. and then to half of the suspensions 0·1 ml. of 0·11 M-$CaCl_2$ was added. Incubation of the suspensions was continued and at various intervals tubes containing calcium and tubes without calcium were cooled in ice and centrifuged at 0° at 3000 rev./min. for 3 min. The trichloroacetic acid-insoluble N content of the supernatant was determined.

In other experiments leucocytes were treated in the same way but the calcium was added before, or for various periods after, addition of the leucocidin and incubation at 37° was continued for 7 min. after addition of the calcium. The cell suspensions were centrifuged at 0° and the supernatants collected. The trichloroacetic acid-insoluble N contents and the β-glucuronidase or the aldolase contents of the supernatants were determined.

To determine the effect of nucleoside derivatives in maintaining the ability of leucocidin-treated leucocytes to extrude β-glucuronidase when treated with calcium, 10^8 leucocytes in 3 ml. of calcium-free Hanks medium were added to tubes, some of which contained nucleoside derivatives dissolved in 0·2 ml. of 0·9% NaCl solution. Leucocidin was then added and the tubes were incubated at 37° for 5 min. Calcium (0·03 ml. of 0·11 M-$CaCl_2$) was then added and incubation continued for 7 min. The tubes were then centrifuged at 0° and the β-glucuronidase content of the cell supernatant was determined. To determine the effect of ATP on the release of aldolase from leucocidin-treated leucocytes in calcium-free media, tubes were prepared in the same way but no calcium was added. The aldolase content of the cell supernatants was determined.

To determine the effect of antileucocidin antibody on the extrusion of protein from the leucocidin-treated leucocyte two suspensions of 2×10^8 cells in 5 ml. of calcium-free Hanks solution were incubated with leucocidin (F component 1·5 L^+ units and S component 9·0 L^+ units) for 5 min. at 37°. [The definition of the L^+ unit and the unit of the antileucocidin antibody is given by Woodin (1961).] To one suspension 10 units of antibody were added and then to both suspensions 0·075 ml. of 0·11 M-$CaCl_2$ was added. The two suspensions were incubated for a further 7 min. and centrifuged. The β-glucuronidase content of the supernatant was determined.

Effect of calcium on the ATP and inorganic phosphate content of the leucocidin-treated leucocyte. Tubes were prepared with 8×10^8 leucocytes in 3·0 ml. of calcium-free, phosphate-free Krebs-Ringer bicarbonate solution. To one series of tubes 0·05 ml. of 0·11 M-$CaCl_2$ and 0·02 ml. of 4% (w/v) Ca–EDTA, pH 7·2, were added. To the other series of tubes 0·02 ml. of 4% (w/v) Mg–EDTA, pH 7·2, was added. The solutions of EDTA were adjusted to pH 7·2 with 50% (w/v) NaOH. The cell suspensions were then incubated at 37° with added leucocidin. After 3 min. two tubes containing calcium, and two tubes without calcium, were cooled in ice. To one tube containing calcium and one tube without calcium 0·3 ml. of 100% (w/v) trichloroacetic acid was added. The other two tubes were centrifuged at 2000 rev./min. for 3 min. and the supernatants collected. The cell pellets were extracted with 3 ml. of ice-cold 10% (w/v) trichloroacetic acid and the volume of the extract was noted. Further tubes that had been incubated for 7 min. and for 10 min. were treated in the same way. The cell suspensions and cell pellets were extracted with trichloroacetic acid for 30 min. and centrifuged at 3000 rev./min. for 5 min. The supernatants were collected and shaken four times with an equal volume of ether and the ether layers rejected. The aqueous layers were centrifuged at 3000 rev./min. and the supernatants analysed for their ATP and inorganic phosphate contents. In calculating the total ATP and inorganic phosphate contents of the cell suspensions and the cell pellets, the anhydrous volume of the insoluble cell material was neglected. The β-glucuronidase content of the cell supernatants was determined.

Distribution of β-glucuronidase, ribonuclease and aldolase in subcellular fractions of the leucocyte. Duplicate suspensions of 10^9 leucocytes were prepared in 5 ml. of calcium-free Hanks medium and then 0·075 ml. of 0·11 M-$CaCl_2$ was added to one suspension. Both suspensions were incubated for 10 min. at 37° with leucocidin, centrifuged at 0° and the supernatants collected. The cell pellets were then homogenized by the procedure of Cohn & Hirsch (1960). Complete breakage of the cells in the absence of calcium was obtained in a few minutes, but to achieve complete breakage of the cells in the suspension containing calcium it was necessary to homogenize in a Potter–Elvehjem tube for 15 min. The homogenates were diluted to 10 ml. with 0·32 M-sucrose and centrifuged at 30 000 rev./min. for 1 hr. The supernatant, which was clear, was collected and the insoluble fraction suspended in 5 ml. of 0·32 M-sucrose. The enzyme contents of the cell supernatant and the soluble and insoluble fraction of the homogenates were determined.

Preparation of the granule fractions of normal and leucocidin-treated leucocytes. Suspensions of 2×10^8 leucocytes in 10 ml. of calcium-free Hanks solution were incubated at 37° alone or with added leucocidin for 10 min. They were centrifuged and the supernatants rejected. The cell pellets were homogenized and the granule fractions were prepared by the method of Cohn & Hirsch (1960). Samples (3 ml.) of the granule fractions (20 ml. in 0·32 M-sucrose) were taken for the determination of the trichloroacetic acid-insoluble N content and further samples (0·5 ml.) were used for the determination of the nucleoside-triphosphatase activity.

Estimation of enzyme activities. The activity of the nucleoside triphosphatase of the granule fraction was observed in mixtures containing, in 1·5 ml., 100 μmoles of tris–HCl, pH 7·5, 1·0 μmole of ATP or GTP (sodium salts), 5·5 μ-

moles of $CaCl_2$ or $MgSO_4$, 150 μmoles of NaCl, 7·7 μmoles of KCl and 0·5 ml. of the granule fraction in 0·32 M-sucrose. The mixtures were incubated at 37° for 30 min., diluted with 5 ml. of ice-cold 0·32 M-sucrose and centrifuged at 15000 rev./min. for 30 min. The orthophosphate in the supernatant was determined. Ribonuclease was determined by the method of Cohn & Hirsch (1960). β-Glucuronidase was determined by the method of Fishman, Springer & Brunetti (1948). The unit of β-glucuronidase used by us is the amount of enzyme that liberates 1 μg. of phenolphthalein under the conditions of the assay. Aldolase was determined by the method of Wu & Racker (1959). The unit of aldolase used by us is the amount of enzyme that decomposes 1 μmole of fructose 1,6-diphosphate in 1 min.

Estimation of ATP. This was done spectrophotometrically, glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase being used. Mixtures containing, in 3 ml., 10 μmoles of 3-phosphoglycerate, 750 μmoles of tris–HCl, pH 7·5, 30 μmoles of cysteine–HCl, 40 μmoles of Mg–EDTA, 15 μmoles of $MgSO_4$, 0·3 μmole of NADH, 80 μg. of glyceraldehyde phosphate dehydrogenase, 20 μg. of 3-phosphoglycerate kinase (C. F. Boehringer und Soehne, Mannheim, Germany) and 0·4–0·8 ml. of leucocyte extract were incubated at room temperature and the decrease in extinction at 340 mμ was determined.

Chemical methods. Calcium and magnesium were determined by the methods of Naora, Naora, Mirsky & Allfrey (1961). Inorganic phosphate was determined by the method of Berenblum & Chain (1938). Nitrogen was determined by micro-Kjeldahl procedure. The protein content was calculated from the trichloroacetic acid-insoluble N content, assuming the protein to contain 15% of N.

Determination of radioactivity. Samples containing less than 1 mg. were dried on planchets (surface area 3·9 cm.^{2}) and taken as being at infinite thinness. They were counted with a mica end-window Geiger counter.

RESULTS

Accumulation of calcium and loss of magnesium by the leucocidin-treated leucocyte. Table 1 shows that there is an accumulation of calcium in and a loss of magnesium from the leucocyte when it is treated with leucocidin. The measurements recorded in Table 1 were made on the centrifuged cell pellets. Determination of the extracellular volume from the amount of [^{131}I]albumin trapped in the cell pellet gave the values 25 and 55% for normal and leucocidin-treated leucocytes respectively. These values were used to calculate the concentration and content of calcium and magnesium inside the cell.

It was found that the leucocidin-treated cell accumulated calcium when it was added at 0° (Table 2). The leucocidin-treated cell is thus freely permeable to calcium and the accumulation is due to increased adsorption by the cell. It was of interest to determine the amount adsorbed as a function of the calcium concentration in the cell supernatant and of the time at which the leucocidin-treated cells were incubated in a calcium-

Table 1. *Calcium and magnesium content of normal and leucocidin-treated leucocytes*

Concentrations of calcium and magnesium in the cell water were obtained by taking the extracellular volume in the cell pellets as 25% and 55% for normal and leucocidin-treated cells respectively. Results are the means of nine determinations on cells incubated in ascitic fluid and two determinations on cells incubated in Hanks medium.

Material	Concn. of calcium (μg./ml. of cell water)	Calcium content (μg./mg. dry wt.)	Concn. of magnesium (μg./ml. of cell water)	Magnesium content (μg./mg. dry wt.)	Concn. of calcium in cell supernatant (μg./ml.)	Concn. of magnesium in cell supernatant (μg./ml.)
Normal leucocytes in ascitic fluid	112	0·34	188	0·6	76	15
Leucocidin-treated leucocytes in ascitic fluid	440	1·6	126	0·25	63	16
Normal leucocytes in Hanks medium	100	0·85	—	—	67	—
Leucocidin-treated leucocytes in Hanks medium	740	2·6	—	—	67	—

Table 2. *Accumulation of calcium by leucocidin-treated leucocytes at* 0°

Duplicate suspensions of 3×10^7 leucocytes in 5 ml. of calcium-free Hanks solution (containing 18 μg. of magnesium/ml.) were incubated for 10 min. at 37°. One suspension was maintained at 37° and one cooled at 0°; 350 μg. of calcium was added to each. After 10 min. the cell pellets were analysed.

Conditions	Dry wt. of pellet (mg.)	Wet wt. of pellet (mg.)	Calcium in pellet (μg.)	Calcium adsorbed (μg./mg. dry wt.)
Calcium added at 0°	20	255	47	1·60
Calcium added at 37°	18·2	292	48	1·65

free medium. The amount adsorbed was calculated by assuming that the concentration of Ca^{2+} ions in solution in the total water of the centrifuged cell pellet was the same as that in the cell supernatant and the excess in the cell pellet was taken as the amount adsorbed by the cell. Table 3 shows that the amount of calcium adsorbed by the leucocidin-treated leucocyte is reduced as the period of incubation in calcium-free solution is increased. It will be shown below that the amount of protein released from the granules is also reduced under these conditions, but from the amount of calcium adsorbed and that required to induce protein extrusion (see below) it appears that it is not a deficiency in adsorption of calcium that is limiting in these conditions.

In a further experiment, normal and leucocidin-treated cell pellets were homogenized and the disintegrated cells were dialysed against Hanks medium. Table 4 shows that there is an accumulation of calcium by disintegrated normal or leucocidin-treated leucocytes. During preparation of the homogenate of the cells used in this experiment the cell supernatant was first removed. The supernatant of the leucocidin-treated cell contains the protein derived from the granules (1·5 mg./10^8 cells; Woodin, 1961) and the dry weight of this does not contribute to the dry weight reported in Table 4. Corrected for the weight of the protein lost into the supernatant, the dry weight of the organic material (74 mg.) of the leucocidin-treated cells described in Table 4 becomes 89·8 mg., and this gives the amount of calcium adsorbed as 1·48 μg./mg. dry wt. Thus treatment with leucocidin does not increase the number of binding sites associated with the non-diffusible structures of the cell.

Dependence of the extrusion of protein by the leucocidin-treated leucocyte on the presence of calcium. In the absence of calcium the amount of protein released from the leucocidin-treated leucocyte was greatly reduced. Fig. 1 shows that when calcium is added to leucocytes after addition of leucocidin there is a stimulated output of protein, although the amount released is smaller than that released by leucocidin from leucocytes prepared in media containing calcium (1·5 mg./10^8 cells; Woodin, 1961). There was considerable variation in the amount of protein present in the supernatants of cells treated with leucocidin in the absence of calcium (Table 5) and the amount is in excess of that present in the supernatant of normal cells in a calcium-free medium. In no case was there significant β-glucuronidase in the supernatant of cells treated with leucocidin in calcium-free media (Table 5), and in subsequent experiments to determine the conditions necessary for the extrusion of the contents of the granules, effects were assessed from the amount of β-glucuronidase appearing in the cell supernatant.

Table 6 gives the subcellular distribution of ribonuclease and β-glucuronidase, two enzymes that are known to be present in the granules of the polymorphonuclear leucocyte (Cohn & Hirsch, 1960). There is no release of ribonuclease or β-glucuronidase into the cytoplasm of the leucocidin-treated cell in the absence of calcium. Electron micrographs of leucocidin-treated cells prepared in calcium-free media show intact granules (Woodin, French & Marchesi, 1963).

The amount of β-glucuronidase released from the leucocidin-treated leucocyte is dependent upon the time at which the calcium is added. Addition of calcium to a suspension of leucocidin-treated

Table 3. *Effect of incubation in a calcium-free medium on the subsequent adsorption of calcium by leucocidin-treated leucocytes*

Suspensions of 3×10^8 leucocytes in 5 ml. of calcium-free Hanks medium (containing 18 μg. of magnesium/ml.) were incubated at 37° with leucocidin and 560 or 355 μg. of calcium was added at the time indicated. After a further incubation for 10 min. the cell pellets were analysed.

Incubation period before addition of calcium (min.)	Calcium adsorbed (μg./mg. dry wt.) Concn. of calcium in cell supernatant 110 μg./ml.	Concn. of calcium in cell supernatant 70 μg./ml.
0	2·5	1·85
3	2·0	1·55
8	1·8	1·40

Table 4. *Adsorption of calcium by disintegrated leucocytes*

Cell pellets of centrifuged normal and leucocidin-treated leucocytes were homogenized and the homogenates were dialysed against Hanks solution at 0° (containing 67 μg. of calcium/ml.). The dialysed homogenates were analysed.

Material	Dry wt. (mg.)	Water content (ml.)	Calcium content (μg.)	Dry wt. of organic material (mg.)	Calcium adsorbed (μg./mg. dry wt. of organic material)
Homogenate of normal cells	56	0·41	102	52·6	1·5
Homogenate of leucocidin-treated cells	86	1·2	212	74	1·8

leucocytes incubated for 5 min. in a calcium-free medium induced the extrusion of only about 50% of the amount extruded when the calcium was added before the leucocidin (Table 5). There was no extrusion of β-glucuronidase if calcium was added to leucocidin-treated leucocytes at 0°. Table 7 describes the effect of increasing calcium concentration on the amount of β-glucuronidase released. The calcium adsorbed at the calcium concentration giving the maximal response is similar to that adsorbed in the cells incubated for several minutes in calcium-free media (see Table 3). The submaximal release of β-glucuronidase under the latter conditions is not due to a deficiency in the amount of adsorbed calcium.

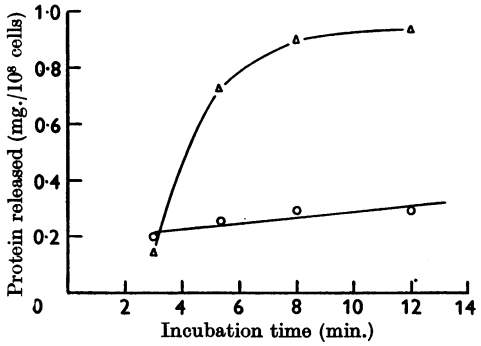

Fig. 1. Release of protein induced in leucocidin-treated leucocytes by calcium. Leucocidin was added at zero time and incubation carried out at 37°. ○, No calcium added; △, calcium (67 μg./ml.) added after 3 min. at 37°.

Addition of antileucocidin to leucocytes incubated for 7 min. with leucocidin in calcium-free Hanks solution did not decrease the amount of β-glucuronidase released by subsequent addition of calcium.

Increased permeability of the leucocidin-treated leucocyte in the absence of extracellular calcium. It has been noted above that the amount of protein released from the leucocidin-treated leucocyte, although containing little β-glucuronidase, is still in excess of that in the supernatant of normal cells incubated under similar conditions. The super-

Table 5. *Release of protein and β-glucuronidase on addition of calcium to leucocidin-treated leucocytes*

Leucocytes suspended in calcium-free Hanks solution (containing 18 μg. of magnesium/ml.) were incubated with leucocidin and calcium was added as indicated. Suspensions were incubated at 37° for 7 min. after the addition of calcium (67 μg./ml.) or, where no calcium was added, for 10 min. after the addition of leucocidin. The protein and β-glucuronidase in the cell supernatant were determined.

Expt. no.	Incubation conditions	Total protein in supernatant (mg./10⁸ cells)	β-Glucuronidase in supernatant (units/10⁸ cells)
1	No Ca^{2+} ions	0·35	8
	Ca^{2+} ions added at start of incubation	0·97	120
2	No Ca^{2+} ions	0·28	9·1
	Ca^{2+} ions added at start of incubation	0·35	81·0
	Ca^{2+} ions added after incubation for 3 min.	0·55	63
3	No Ca^{2+} ions	0·52	7·0
	Ca^{2+} ions added at start of incubation	0·62	93·0
	Ca^{2+} ions added after incubation for 5 min.	0·47	48·0
4	No Ca^{2+} ions added	—	13
	Ca^{2+} ions added at start of incubation	—	83
	Ca^{2+} ions added after incubation for 7 min.	—	14

Table 6. *Effect of extracellular calcium on the subcellular distribution of some enzymes in leucocidin-treated leucocytes*

Suspensions of 10^9 leucocytes in 10 ml. of Hanks solution (containing 18 μg. of magnesium/ml. and 67 μg. of calcium/ml.) or in 10 ml. of calcium-free Hanks solution (containing 18 μg. of magnesium/ml.) were incubated with leucocidin and the cell supernatant was collected. The cell pellets were homogenized. The soluble fraction of the homogenate is the supernatant from centrifuging the homogenate at 30 000 rev./min. for 1 hr.

Fraction	Calcium in cell suspension	Ribonuclease (% amount recovered)	β-Glucuronidase (% amount recovered)	Aldolase (% amount recovered)
Cell supernatant	Present	79	72	2·0
	Absent	6·1	1·0	14·0
Soluble fraction of homogenate	Present	8·6	2·0	85
	Absent	5·1	3·0	76
Insoluble fraction of homogenate	Present	12·4	26	13
	Absent	88·8	96	10

natant of the leucocidin-treated leucocytes in calcium-free media is rich in aldolase. Table 6 indicates that this enzyme is present in the soluble fraction of the cytoplasm and its presence in the supernatant of the leucocidin-treated leucocyte in the absence of extracellular calcium is ascribed to leakage. Table 9 shows that there is a decreased retention of orthophosphate in the leucocidin-treated leucocyte when calcium is omitted from the medium. Table 7 shows that increasing concentrations of calcium in the suspensions of leucocidin-treated cells decreased the amount of aldolase released; it appears that, simultaneously to inducing extrusion of the contents of the granules, calcium affords protection against leakage of the soluble contents of the cytoplasm.

Table 7. *Effect of increasing extracellular calcium concentration on the adsorption of calcium and the release of β-glucuronidase and aldolase from leucocidin-treated leucocytes*

Incubated suspensions were 2×10^8 leucocidin-treated leucocytes in 5 ml. of calcium-free Hanks solution (containing 18 μg. of magnesium/ml.) to which calcium had been added to give the concentrations indicated. The cell pellets were isolated and the adsorbed calcium was determined; the aldolase and β-glucuronidase in the cell supernatant were determined.

Calcium added to cell suspension (μg./ml.)	Calcium adsorbed (μg./mg. dry wt.)	β-Glucuronidase in cell supernatant (total units)	Aldolase in cell supernatant (total unit)
0	56	17	0·102
0·8	65	120	0·087
4·4	150	265	0·010
8·8	150	270	0·007
17·0	160	278	0·007
44·0	140	278	0·007

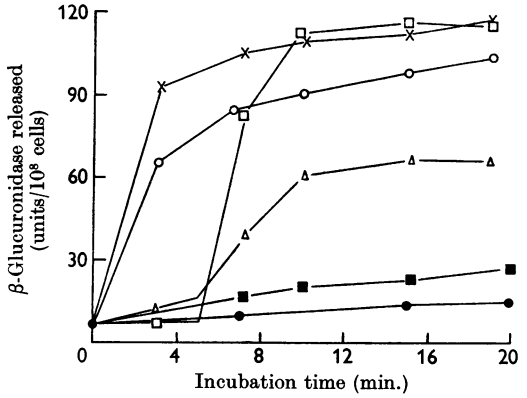

Fig. 2. Effect of ATP in preserving the ability of leucocidin-treated leucocytes to extrude β-glucuronidase when treated with calcium. Leucocidin was added to leucocytes in calcium-free Hanks solution (containing 18 μg. of magnesium/ml.) at zero time and incubation was at 37°. ×, ATP (0·6 μmole/ml.) and calcium (67 μg./ml.) were added at zero time; ○, calcium (67 μg./ml.) was added at zero time; □, ATP (0·6 μmole/ml.) and calcium (67 μg./ml.) were added after incubation for 5 min.; △, calcium (67 μg./ml.) was added after incubation for 5 min.; ●, ATP (0·6 μmole/ml.) was added but no calcium; ■, no calcium or ATP was added. In constructing the curve for the points □ and the curve for the points △ a linear release of β-glucuronidase in the period 0–5 min. is assumed.

Influence of nucleoside triphosphates in stimulating the extrusion of protein induced by calcium in the leucocidin-treated leucocyte. It has been recorded above that incubation of the leucocidin-treated leucocyte in a calcium-free medium reduces the amount of β-glucuronidase released on subsequent addition of calcium. Incubation in the presence of ATP or GTP prevents this reduction in the efficiency of calcium in inducing β-glucuronidase extrusion. The effect of ATP is illustrated in Fig. 2 and Table 8 records the effect of some similar compounds.

ATP (final concn. 0·6 μmole/ml.) produced a fourfold decrease in the amount of aldolase released from the leucocidin-treated leucocyte incubated for 10 min. in calcium-free Hanks medium.

Table 9 shows that the extrusion of β-glucuronidase induced in the leucocidin-treated leucocyte by calcium is not associated with an increased hydrolysis of ATP or with an increased accumulation of inorganic phosphate.

Presence of a nucleoside triphosphatase in the granules of the leucocyte. It was observed that there was rapid hydrolysis of ATP or GTP by the isolated granules of the leucocyte. This effect is

Table 8. *Restoration of the ability of calcium to induce the extrusion of β-glucuronidase from leucocidin-treated leucocytes*

Suspensions of 10^8 leucocidin-treated leucocytes in 3 ml. of calcium-free Hanks medium (containing 18 μg. of magnesium/ml.), with the compounds listed below, were incubated for 7 min. Calcium (220 μg.) was then added and incubation continued for 5 min. The β-glucuronidase in the cell supernatant was determined. Stimulated extrusion of β-glucuronidase is the ratio: β-glucuronidase extruded in the presence of added compound/β-glucuronidase extruded in the absence of the added compound.

Addition to medium (μmoles/ml.)	Stimulated extrusion of β-glucuronidase
ATP (0·6)	4·5
ADP (0·6)	4·5
AMP (1·2)	1·5
Adenosine (1·3)	1·3
GTP (0·6)	4·5
ITP (0·6)	2·6

Table 9. *Effect of incubation with calcium on the content and retention of adenosine triphosphate and inorganic phosphate in leucocidin-treated leucocytes*

Suspensions of 7×10^8 leucocytes in 3 ml. of calcium-free, phosphate-free Krebs–Ringer bicarbonate solution (containing 29 μg. of magnesium/ml.) were incubated with leucocidin and the additions listed below. The ATP and inorganic phosphate of the cell suspension and of the cell pellet obtained by centrifuging at 0° were determined. The β-glucuronidase in the cell supernatant was determined.

Addition	Time of incubation (min.)	β-Glucuronidase in cell supernatant (units/ml.)	ATP content (μmole)		Inorganic phosphate content (μmoles)	
			In cell suspension	In cell pellet	In cell suspension	In cell pellet
Ca–EDTA (800 μg.)	3	66	0·55	0·55	1·38	0·94
(Ca^{2+} ions 220 μg.)	7	124	0·47	0·43	1·52	1·10
	10	151	0·44	0·42	2·00	1·15
Mg–EDTA (800 μg.)	3	8·0	0·49	0·47	1·42	0·43
	7	9·7	0·38	0·36	2·25	0·54
	10	10·7	0·27	0·25	2·50	0·49

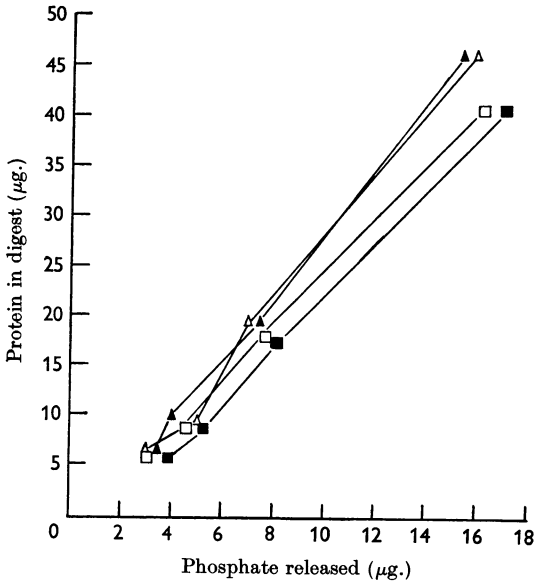

Fig. 3. Nucleoside-triphosphatase activity of granules from the leucocyte. □, Granules from leucocidin-treated leucocytes, activated by calcium (3·65 mM); ■, granules from leucocidin-treated leucocytes, activated by magnesium (3·65 mM); ▲, granules from normal leucocytes, activated by calcium (3·65 mM); △, granules from normal leucocytes, activated by magnesium (3·65 mM).

observed only in the presence of Ca^{2+} or Mg^{2+} ions, which are both maximally effective at 1·2 mM. There was no hydrolysis of ATP by the granules in the presence of dinitrophenol (30 μM). Fig. 3 shows that a small difference can be detected in the amount of hydrolysis effected by the granules from normal or leucocidin-treated cells and that magnesium and calcium are equally effective in activating the process.

DISCUSSION

The intracellular accumulation of calcium is a common occurrence in the injured cell (Cameron & Spector, 1961). In the leucocidin-treated leucocyte the process occurs against a concentration gradient and can take place at 0°. It is most probably due to an increased accessibility of binding sites. Many of these can be afforded by the insoluble structures of the cell but under some conditions the amount of calcium accumulated is in excess of the number of sites they provide. Additional calcium binding could be provided by orthophosphate, which is known to accumulate in the leucocidin-treated cell (Woodin, 1961). The accumulation of calcium in the leucocidin-treated leucocyte is associated with a greatly increased resistance to homogenization and with a decreased permeability to aldolase and to orthophosphate. It is probable that some of the calcium is bound on the surface of the cell. The dependence of the normal impermeability of the mammalian cell on the presence of calcium is well known (Harris, 1960; Maizels, 1960).

In the leucocyte the accumulation of calcium simultaneously protects against loss of the soluble components of the cell and activates the process by means of which the contents of the granules are extruded to the exterior of the cell. The presence of extracellular calcium has been shown to be necessary for secretion in other tissues. The rate of acetylcholine secretion at the nerve ending is critically dependent upon the presence of extracellular calcium (Katz, 1962), and stimulation of release of catecholamine from the adrenal medulla is dependent upon the presence of extracellular calcium and is associated with an increased exchange of extracellular calcium with that of the tissue (Douglas & Rubin, 1961; Douglas & Poisner, 1962). It is probable that secretion in these tissues has features in common with those observed to take

place during protein extrusion in the leucocyte and proceeds through direct fusion of the granules or vesicles with the surface of the cell (Katz, 1962; De Robertis, Nowinski & Saez, 1960).

The transfer of material from granules to the exterior of the cell by a process that does not involve the solubilization of the granule contents in the cytoplasm must involve three phases. There must be a collision of the granule and the surface of the cell and this must be accompanied by an adherence of the granule to the surface that persists long enough for the third phase, the passage of the contents of the granule to the exterior, to occur.

That the collision of the granules and the cell surface is facilitated in the leucocidin-treated leucocyte is apparent from observations with the light-microscope, which show that immediately after treatment of the cell with leucocidin the granules lose their orderly streaming and go into violent brownian motion (Gladstone & van Heyningen, 1957). This effect can be observed if calcium is omitted from the medium (Woodin *et al.* 1963). It is probable that calcium increases the binding sites for the adherence of the granules and the cell surface for addition of calcium to leucocidin-treated cells at 0° stops the brownian movement of the granules, many of which can be seen to be attached to the surface (Woodin *et al.* 1963). The possibility that calcium is also involved in the third phase, the passage of the contents to the exterior, cannot be excluded.

Leucocidin does not play a direct part in the extrusion of the contents of the granules, for addition of calcium to the leucocidin-treated leucocyte after neutralization of leucocidin by antibody induces the release of β-glucuronidase. It is probable that the normal leucocyte is equipped with the reagents required for the fusion of the granules with the cell surface. The normal leucocyte is typically phagocytic, a process that involves the fusion of opposing parts of the cell surface. Similarly, after phagocytosis and the transfer of the phagocytosis vesicle to the cell interior there is a loss of the granules of the cell with retention of the granule contents within the cytoplasm (Hirsch & Cohn, 1960), a process that may involve fusion of the surface of the granule with that of the phagocytosis vesicle.

The role of the nucleoside phosphates in the extrusion of the contents of the granules is not clear. It has been shown that the extrusion induced by calcium is not associated with a stimulated ATP breakdown or accumulation of orthophosphate. There is a reduced rate of glycolysis in the leucocidin-treated cell and evidence has been presented that the increased incorporation of ^{32}P into nucleotides is not a result of increased synthesis (Woodin & Wieneke, 1963). Thus the extrusion is not dependent upon the continuous regeneration of nucleoside phosphates. However, as ATP breakdown does occur during extrusion it cannot be concluded that the two processes are independent.

The amount of protein extruded from the granules is reduced as the period of incubation of the leucocidin-treated leucocyte in a calcium-free medium is increased. This effect can be prevented if nucleoside phosphates are added to the calcium-free medium. This cannot be taken as evidence that these substances participate directly in the morphological changes or the chemical reactions associated with the extrusion of the protein from the granules. It has been shown that adenosine triphosphate protects the leucocidin-treated cell against the loss of aldolase that occurs in a calcium-free medium and this suggests that the added nucleoside phosphates preserve the structure of the cell surface. It is possible that it is by a similar preservation of structure that the nucleoside phosphates enable the leucocyte to retain its ability to extrude protein when stimulated by calcium.

SUMMARY

1. After treatment with leucocidin there is a loss of magnesium from and a gain of calcium by the leucocyte. The accumulation of calcium occurs if the calcium is added to the leucocidin-treated cell at 0°.

2. Mechanically disintegrated leucocytes adsorb calcium from solution. There is no difference in the amount adsorbed by disintegrated normal or leucocidin-treated cells.

3. When calcium is omitted from the medium the extrusion of protein from the granules of the leucocidin-treated leucocyte is inhibited and the cell simultaneously becomes more permeable to orthophosphate and to aldolase.

4. When calcium is added to leucocidin-treated leucocytes suspended in calcium-free media the release of β-glucuronidase from the granules is induced. The amount released decreases as the period of incubation in calcium-free media is increased.

5. The loss of efficiency of calcium in inducing extrusion of β-glucuronidase can be prevented if nucleoside phosphates are added to the leucocytes in calcium-free media.

6. There is no increased hydrolysis of adenosine triphosphate or accumulation of orthophosphate in the leucocidin-treated leucocyte when extrusion of β-glucuronidase is induced by adding calcium.

7. The presence of a nucleoside triphosphatase in the granules of the leucocyte is described. This is activated by calcium or magnesium and small differences can be detected in the activity of granules from normal or leucocidin-treated cells.

8. The role of calcium, leucocidin, nucleoside phosphates and the equipment of the normal leucocyte in the process of extrusion of protein from the granules is discussed.

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The Distribution of Nucleotides in Deoxyribonucleic Acid

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Deoxyribonucleic acid is degraded by diphenylamine in acid solution to pyrimidine oligonucleotides of the general formula Py_np_{n+1}, where Py represents pyrimidine deoxynucleoside and p is phosphate (Burton, 1956; Burton & Petersen, 1957, 1960). The scission of the backbone chain requires an aromatic amine in addition to weak acid (Burton & Petersen, 1960) and the ultimate products are the same as the principal products of hydrolysis by mineral acids under more drastic conditions (Levene & Jacobs, 1912; Dekker, Michelson & Todd, 1953; Cohn & Volkin, 1957; Shapiro & Chargaff, 1957*a*, *b*). After terminal dephosphorylation with prostatic phosphomonoesterase, a number of these products can be separated by two-dimensional paper chromatography. In this way, the frequencies of occurrence of pyrimidine nucleotides in deoxyribonucleic acid derived from several sources have been determined (Burton & Petersen, 1960; Burton, 1960).

Petersen (1961) separated many more of the products by means of two-dimensional electrophoresis and chromatography on paper, as applied by Rushizky & Knight (1960*a*, *b*) to the separation of the products of pancreatic ribonuclease action. This paper describes further improvements in the method and gives the results obtained from the degradation of deoxyribonucleic acid from several sources.

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MATERIALS AND METHODS

Deoxyribonucleic acid. The calf-thymus DNA used in this study was the same preparation as described by Burton & Petersen (1960). Herring-testis DNA, prepared by the method of Emanuel & Chaikoff (1953), was a gift from Dr M. R. Lunt. Samples of diphenylamine-degraded DNA of *Alcaligenes faecalis* and *Pseudomonas aeruginosa* were those used by Burton (1960).

Phosphorus estimations. Inorganic and total phosphate of DNA digests and of material eluted from paper chromatograms were determined as described by Burton & Petersen (1960).

Paper for chromatography and electrophoresis. Sheets of Whatman no. 1 paper (47 cm. × 57 cm.) were washed chromatographically, six at a time, with N-HCl (2 l.) and water (3 l.) and dried at room temperature.

Paper electrophoresis. A Perspex apparatus, essentially the same as that described by Rushizky & Knight (1960*a*), was used. The formate buffer used in the apparatus was 2·5 times as concentrated as that used by Rushizky & Knight (1960*a*) and was prepared by adding 45 ml. of 98–100% formic acid to 6 l. of water and titrating to pH 2·7 at room temperature with approx. 8 ml. of aq. NH_3, sp.gr.