comparison of the means of the two groups of cystine coefficients indicated that the probability that the two sets of data belong to different populations is very low ($P < 0.01$), which suggests that a full range of cystine excretion from normal to high can be expected in dog populations. Since canine cystinuria is inherited (Kassell, Brand & Cahill, 1940), an incompletely recessive mode of inheritance would be indicated to explain the existence of normal, intermediate and very high levels of cystine excretion in the same population.

SUMMARY

1. A method for the estimation of cystine as cysteic acid in urine and plasma is described.

2. Cystine concentrations in the urine of 11 dogs with cystine urolithiasis were compared with those of 12 clinically normal dogs. The values have been corrected for differing urine concentrations by dividing by the urinary creatinine in g./l. to give the 'cystine coefficient'.

3. The cystine-urolithiasis group had cystine coefficients between 61 and 600 mg. of cystine/g. of creatinine, whereas the normal animals had coefficients between 11 and 210. Factors other than the level of cystine excretion may be important in stone formation. There was a low statistical probability that the two groups belonged to different populations ($P < 0.01$).

4. Plasma cystine concentrations in seven dogs with cystine-stone disease were not significantly higher than the seven values obtained from normal animals. This supports the theory that the cystinuria of canine cystine-stone disease is renal in origin.

The author is grateful to the staff of the Liverpool Veterinary Hospital and to the several dog owners and veterinary practitioners who provided specimens and information. Thanks are also due to Professor E. G. White for his guidance and criticism and to the Wellcome Trust for a grant which financed this work. The work forms part of a Ph.D. Thesis submitted to the University of Liverpool.

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The Participation of Calcium, Adenosine Triphosphate and Adenosine Triphosphatase in the Extrusion of the Granule Proteins from the Polymorphonuclear Leucocyte

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When polymorphonuclear leucocytes are treated with staphylococcal leucocidin in the presence of calcium, proteins known to be present in the cytoplasmic granules are released into the medium, whereas proteins that are found in solution when the cells are homogenized are retained within the cell. The extrusion of the contents of the granules is achieved by a process that involves fusion of the granule with the cell membrane. The omission of calcium from the medium in which the cells are treated with leucocidin inhibits the extrusion of the granule proteins and simultaneously increases the permeability of the cell to the soluble constituents of the cytoplasm (Woodin & Wieneke, 1963*b*; Woodin, French & Marchesi, 1963).

The addition of calcium to leucocytes treated with leucocidin in a calcium-free medium induces the extrusion of the granule proteins, but the

amount released in this way is decreased by prolonging the incubation in calcium-free media. This loss of response of the leucocidin-treated leucocyte to calcium can be overcome by adding certain nucleoside phosphates. If leucocidin is neutralized by antibody before the addition of calcium the extrusion of the granule proteins is not affected, and this suggests that leucocidin does not play a direct part in the extrusion process. It is possible to distinguish the primary cytotoxic effect of leucocidin from a secondary effect, the extrusion process, which is a response of the leucocidin-treated cell to calcium. The secondary effect is dependent on the presence of nucleoside phosphates.

The present paper extends the observations on the function of calcium and nucleoside phosphates in the extrusion process. It is also shown that the extrusion of the granule proteins can follow treatment of the leucocyte with vitamin A, and this process is also dependent on the presence of calcium and ATP.

MATERIALS AND METHODS

Materials. Leucocidin, polymorphonuclear leucocytes (referred to in this paper as leucocytes), the media for suspending the cells and ^{131}I-labelled albumin were obtained and used as described by Woodin & Wieneke (1963*a*, *b*). Unless otherwise stated, the concentration of the leucocytes was 5×10^7/ml. Vitamin A alcohol was obtained from Roche Products, London. It was dissolved in ethanol (1 g./ml.) and stored at $-12°$ under nitrogen. EDTA (magnesium salt) (L. Light and Co. Ltd., Colnbrook, Bucks.) was used as a 4% (w/v) solution in water, adjusted to pH 7·2 with 10N-NaOH. [^{32}P]ATP (sodium salt) was prepared by the method of Colowick & Kaplan (1957). Unlabelled ATP (sodium salt) was obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Release of granule enzymes from leucocytes treated with vitamin A. Leucocytes in 1 ml. of Hanks medium or calcium-free Hanks medium were added to tubes containing 10 μl. of vitamin A alcohol in ethanol (40 mg./ml.) and immediately mixed by shaking. They were incubated at 37° for 10 min. and the enzyme content of the cell supernatant was determined.

In other experiments 10 μl. of vitamin A alcohol in ethanol (40 mg./ml.) was added to tubes that were cooled in ice. Then 1 ml. of leucocytes in ice-cold calcium-free Hanks solution containing 100 μg. of EDTA (magnesium salt)/ml. was added to each tube and immediately mixed by shaking. The tubes were maintained at 0° for 10 min. To some tubes calcium (10 μl. of 0·11M-$CaCl_2$) was added and to some tubes ATP (0·1 or 0·05 ml. of 16·5 mM-ATP) was added. The tubes were then incubated at 37° for 10 min. and the enzyme content of the cell supernatant was determined.

Stimulation of the release of β-glucuronidase, ribonuclease and peroxidase from cells treated with leucocidin. Tubes containing 1 ml. of leucocytes in calcium-free Hanks medium containing 100 μg. of EDTA (magnesium salt)/ml. were incubated with leucocidin at 37° for 10 min., and then to some tubes 10 μl. of 0·11M-$CaCl_2$ and to some tubes 0·1 or 0·05 ml. of 16·5 mM-ATP were added, and incubation was continued for a further 10 min.; the enzyme content of the cell supernatant was determined.

Effect of calcium concentration on the extrusion of granule enzymes from the leucocidin-treated leucocyte. Leucocytes were prepared in phosphate-free calcium-free Krebs–Ringer bicarbonate solution containing $MgSO_4$ (1·18 mM), and 1 ml. volumes were added to tubes. Leucocidin and different amounts of calcium (11 mM- or 0·11M-$CaCl_2$) were added, the tubes incubated at 37° for 10 min., and the enzyme content of the cell supernatant was determined.

In another experiment 1 ml. of leucocytes in phosphate-free calcium-free Krebs–Ringer bicarbonate solution, containing $MgSO_4$ (1·18 mM) and EDTA (magnesium salt) (100 μg./ml.), were incubated with leucocidin for 10 min., and then 0·1 ml. of 16·5 mM-ATP and different amounts of 11 mM- or 0·11M-$CaCl_2$ were added and incubation was continued for a further 10 min. The enzyme content of the supernatant was determined.

Effect of magnesium concentration on the extrusion of β-glucuronidase from the leucocidin-treated leucocyte. Leucocytes in 1 ml. of calcium-free Hanks solution containing 100 μg. of EDTA (magnesium salt)/ml. were incubated with leucocidin for 10 min., and then different amounts of calcium and ATP were added and incubation was continued for 10 min. at 37°. The enzyme content of the cell supernatant was determined.

Tests for the inhibition of the extrusion of proteins. Tubes containing leucocytes in 1 ml. of calcium-free Hanks solution containing 100 μg. of EDTA (magnesium salt)/ml. were incubated with leucocidin for 10 min. at 37°, and then the inhibitor in 0·01 ml. of ethanol or 0·05 ml. of water was added to three tubes. To one of these no further additions were made, to another 25 μl. of 40 mM-ATP was added, and to this and to the third tube 25 μl. of 83 mM-$CaCl_2$ was added. The tubes were incubated for a further 10 min. and the enzyme content of the cell supernatant was determined.

Comparison of different media for the extrusion of proteins in the leucocidin-treated leucocyte. To test for competition between potassium and calcium in the extrusion process leucocytes in 1 ml. of calcium-free Hanks solution containing 100 μg. of EDTA (magnesium salt)/ml. were incubated with leucocidin at 37° for 10 min. and centrifuged at 3000 rev./min. (2200 *g*) for 3 min. at room temperature. The supernatants were rejected and the cell pellets suspended in 0·165M-NaCl or 0·165M-KCl containing different amounts of $CaCl_2$. The suspensions were incubated at 37° for 10 min. and centrifuged at 3000 rev./min. for 3 min. at 0°. The β-glucuronidase content of the cell supernatants was determined.

In other experiments 1 ml. volumes of leucocytes in calcium-free Hanks solution containing 100 μg. of EDTA (magnesium salt)/ml. were incubated with leucocidin for 10 min. and centrifuged at room temperature for 3 min. The cell pellets were suspended in the media described in Table 2, incubated at 37° for 10 min., and the β-glucuronidase, ribonuclease or peroxidase released into the cell supernatant was determined.

Uptake of adenosine [^{32}P]triphosphate by leucocidin-treated leucocytes at 0°. Suspensions of 3×10^8 leucocytes in 1·5 ml. of calcium-free Hanks solution containing 100 μg. of EDTA (magnesium salt)/ml. were incubated with leucocidin in weighed tubes at 37° for 10 min. They were then cooled to 0°, and 50 μl. of 0·11M-$CaCl_2$ and various amounts of

1·65 mM-[^{32}P]ATP (80000 counts/min./μmole) added. The mixtures were maintained at 0° for 5 min. and centrifuged at 0° at 5000 rev./min (3500 *g*) for 6 min. The supernatants were collected. The tubes containing the cell pellets were weighed at room temperature, dried at 110° for 18 hr. and weighed at room temperature again. The dry residues were incubated in 0·5 ml. of N-NaOH at 37° for several hours to give an even suspension of the cell residues. Then 0·5 ml. of 0·9 N-HCl was added and samples (0·5 ml.) of the suspension were taken for determination of radioactivity. The radioactivity in 0·5 ml. of the cell supernatant was also determined.

To further samples of cells 1·65 mM-ATP (unlabelled) was added and then, immediately before centrifuging at 0°, 0·5 ml. of ^{131}I-labelled albumin (containing 20000–50000 counts/min.) was added. The cell pellets were treated as described above, and the radioactivity was determined on 0·05 ml. of the supernatant and of the suspension of the dried cell residues.

The extracellular volume in the centrifuged cell pellet was calculated from the amount of trapped albumin and the intracellular water content determined. The ratio of the concentrations of [^{32}P]ATP inside and outside the cell was calculated.

Distribution of radioactivity in the leucocidin-treated leucocyte after stimulation of the extrusion of proteins with adenosine [^{32}P]*triphosphate.* Four tubes containing 8 ml. of leucocytes in calcium-free Hanks solution containing 100 μg. of EDTA (magnesium salt)/ml. were incubated with leucocidin at 37° for 15 min. with the following additions: tube 1, none; tube 2, 0·4 ml. of 36 mM-[^{32}P]ATP; tube 3, 0·2 ml. of 0·11 M-$CaCl_2$; tube 4, 0·4 ml. of 36 mM-[^{32}P]ATP and 0·2 ml. of 0·11 M-$CaCl_2$. The tubes were incubated at 37° for 8 min. and cooled in ice. A sample (0·5 ml.) of each suspension was withdrawn and the β-glucuronidase in the cell supernatant determined. To the remainder of each suspension 0·4 ml. of 60% (w/v) $HClO_4$ solution was added and after 30 min. at 0° the suspensions were centrifuged at 5000 rev./min. for 15 min. The supernatant was collected and the cell residues were washed twice with 3 ml. of ice-cold 3% (w/v) $HClO_4$. The washings were added to the cell supernatant and shaken twice with 5 ml. of ether. The ether extracts were added to the lipid extracts (see below). After ether extraction the aqueous phase was centrifuged at 5000 rev./min. for 5 min. and the clear supernatant collected. This is the 'acid-soluble fraction' of the leucocytes. The small amount of sediment formed during shaking with ether was added to the 'residual insoluble fraction' (see below).

The material remaining insoluble after washing with 3% (w/v) $HClO_4$ was extracted with 50 ml. of chloroform–methanol (2:1, v/v) and centrifuged. The supernatant, with the added ether washings (see above), is the 'lipid fraction' of the leucocytes. The material insoluble in chloroform–methanol was dried in a stream of air and together with the precipitate from the ether extraction of the acid-soluble fraction (see above) was dissolved in 2 N-NaOH to give the 'residual acid-insoluble fraction'. The total radioactivities of the 'lipid fraction' and 'residual acid-insoluble fraction' were determined. The acid-soluble fraction was fractionated into orthophosphate and acid-soluble esterified phosphate by the Berenblum & Chain method (Woodin & Wieneke, 1963*a*) and the radioactivities of the two fractions were determined.

Specific radioactivity of the phospholipids of the leucocyte after stimulation of the extrusion of proteins with adenosine [^{32}P]*triphosphate.* Four tubes with 10 ml. of leucocytes in calcium-free Hanks solution containing 100 μg. of EDTA (magnesium salt)/ml. were incubated with leucocidin for 10 min. at 37° and then the following additions made: tube 1, none; tube 2, 0·5 ml. of 20 mM-[^{32}P]ATP; tube 3, 0·1 ml. of 110 mM-$CaCl_2$; tube 4, 0·5 ml. of 20 mM-[^{32}P]-ATP and 0·1 ml. of 110 mM-$CaCl_2$. The tubes were incubated for a further 10 min. at 37°, cooled in ice and centrifuged at 3000 rev./min. The β-glucuronidase in the cell supernatant was determined. To the cell pellet in tube 3, 0·5 ml. of 20 mM-[^{32}P]ATP was added and the cell pellets of tubes 2, 3 and 4 were each immediately shaken with 50 ml. of chloroform–methanol (2:1, v/v). The suspensions were left at room temperature for 30 min. and centrifuged at 3000 rev./min. for 10 min. The supernatants were filtered to ensure removal of cell residues. The lipids in the supernatants were washed by 'procedure B' of Woodin (1962). The specific radioactivities of the phospholipid fractions were determined. The [^{32}P]ATP added to the leucocytes contained 16400 counts/min./μmole.

Determination of the concentration of vesicles and granules. The comparison of the composition of the vesicles of the leucocidin-treated cell with the granules of the normal cell is difficult because of the complexity of the conversion of the granule of the vesicle (Woodin *et al.* 1963). It is not certain if any component of the granule remains constant in amount, nor is it certain that a vesicle is produced from a single granule; fusion of several small granules could occur. Thus comparison of composition in terms of the dry weight, water content, protein content or lipid content can be misleading. The concentration of the granules and vesicles in sucrose solution has been determined, immediately after their preparation, from their extinction at 600 mμ. The unit granule or vesicle mass used in the present paper is the amount which, suspended in 1 ml. of 0·32 M-sucrose, would have $E^{1\,cm.}_{600\,m\mu}$ 1·0. In determining the concentration of granules or vesicles they were diluted so that the extinction measured was less than 0·2. A suspension containing 1 unit of granules contains 60 μg. of trichloroacetic acid-insoluble nitrogen and 2·6 μg. of phospholipid phosphorus.

Calcium content and orthophosphate content of granules and vesicles. Granules were isolated by the method of Cohn & Hirsch (1960), except that before suspending the cells in sucrose solution they were washed twice in 20 vol. of ice-cold calcium-free Hanks solution. Vesicles were isolated from leucocidin-treated cells after incubation in Hanks solution containing calcium. The method was that of Cohn & Hirsch (1960), except that the use of a Potter–Elvehjem homogenizer tube was required to ensure complete breakage (Woodin, 1962).

For analysis the granules or vesicles were suspended in sucrose and the concentration was determined from the extinction at 600 mμ. The suspensions were centrifuged at 8000*g* for 30 min., and the pellets extracted with 5 ml. of 5% (w/v) trichloroacetic acid and centrifuged at 3000 rev./min. for 15 min. The calcium and orthophosphate in the supernatant were determined. In some experiments the pellets of granules or vesicles were extracted with 5 ml. of water, adjusted to pH 10 with 5 N-NH_3, or with 5 ml. of chloroform–methanol (2:1, v/v) before extraction with 5% trichloroacetic acid.

To determine the effect of added calcium and ATP on the calcium and orthophosphate content of the granules they were incubated at 37° for 15 min. Mixtures contained 1 μmole of $CaCl_2$, 1 μmole of ATP and 27 units of granules in 3 ml. of 0·32 M-sucrose in 0·02 M-tris–chloride buffer, pH 7·2. After incubation the granules were washed twice with 0·32 M-sucrose by centrifuging at 8000g for 20 min. The pellets were extracted with 5 ml. of 5% trichloroacetic acid and the extracts analysed for calcium and orthophosphate.

When granules after trichloroacetic acid extraction were analysed for their calcium content they were ashed in conc. HNO_3.

Release of protein from isolated granules. Granules were prepared by the method of Cohn & Hirsch (1960). Volumes containing 7·5 units were centrifuged at 8000g for 20 min. and the pellets suspended in 3 ml. of the media described in Table 6. When vitamin A was present the suspension of granules was poured into a tube containing 600 μg. of vitamin A in 0·03 ml. of ethanol and mixed by shaking. The granule suspensions were incubated at 37° or maintained at 0° for 10 min., and then centrifuged at 8000g for 20 min. The enzyme and total protein in the supernatants were determined. A further sample of 7·5 units of granules in 3 ml. of water was frozen and thawed six times and the suspension assayed to give the total activities of the granule enzymes.

Adenosine-triphosphatase activity of isolated granules. The granules were prepared by the method of Cohn & Hirsch (1960). The effect of granule concentration was determined in 1·5 ml. of 0·11 M-sucrose containing 100 μmoles of tris–chloride buffer, pH 7·5, 1·0 μmole of ATP, 5·0 μmoles of $MgCl_2$, and granules. The effects of magnesium and calcium were determined in 1·5 ml. of 0·11 M-sucrose containing 100 μmoles of tris–chloride buffer, pH 7·5, 1·0 μmole of ATP, 3·8 units of granules, and $CaCl_2$ or $MgCl_2$. The effects of sodium and potassium were determined in 1·5 ml. of 0·11 M-sucrose containing 100 μmoles of tris–chloride buffer, pH 7·2, 1·0 μmole of ATP, 1·0 μmole of $CaCl_2$ or $MgCl_2$, 2·5 units of granules, and NaCl or KCl. The effect of ATP concentration was determined in 1·5 ml. of 0·11 M-sucrose containing 100 μmoles of tris–chloride, pH 7·2, 1·0 μmole of $CaCl_2$, 2·5 units of granules, and ATP. The effect of pH was determined in mixtures of 1·5 ml. of 0·11 M-sucrose containing 1·3 μmoles of ATP, 1·3 μmoles of $CaCl_2$, 2·5 units of granules and 0·67 ml. of 0·2 M-tris–maleic acid buffer (pH 6–9).

The mixtures were incubated at 37° for 15 min. and cooled in ice. $HClO_4$ was added to give a final concentration of 3% and the suspensions were centrifuged at 3000 rev./min. for 15 min. The orthophosphate in the supernatant was determined.

Analytical methods. The enzyme released from whole cells was determined in the supernatant prepared by centrifuging cell suspensions for 3 min. at 3000 rev./min. at 0°. The assays of β-glucuronidase and ribonuclease were as given by Woodin & Wieneke (1963*b*); the assay of peroxidase was as given by Woodin (1962). The enzyme units used by us were: β-glucuronidase, the amount liberating 1 μg. of phenolphthalein in 1·5 hr.; ribonuclease, the amount producing an increase in $E^{1\,cm.}_{260\,m\mu}$ of 1·0 in 30 min.; peroxidase, the amount producing an increase in $E^{1\,cm.}_{430\,m\mu}$ of 1·0 in 10 min.

Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Phosphorus was estimated and the radioactivity in material containing ^{32}P determined as described by Woodin & Wieneke (1963*a*). The radioactivity in material containing ^{131}I was determined as described by Woodin & Wieneke (1963*b*). Calcium was determined by the method of Naora, Naora, Mirsky & Allfrey (1961).

Electron microscopy. Normal and leucocidin-treated leucocytes in Hanks medium (2 ml.) and leucocidin-treated leucocytes in calcium-free Hanks medium (2 ml.) were incubated at 37° for 3 min. and then cooled to 0°. An equal volume of 0·6% (w/v) $KMnO_4$ in veronal–acetate buffer, pH 7·6 (Pease, 1960), previously cooled to 0°, was added and the cells were fixed at 0° for 30 min. The suspensions were centrifuged at 200 rev./min. for 3 min. at 0°, and the cell pellets dehydrated and embedded in Araldite. Thin sections were cut and stained with lead by the method of Millonig (1961). Some sections of cells treated with leucocidin in the presence of calcium were extracted with 0·1 M-citric acid at room temperature for 20 min., washed with water and stained with lead by the method of Millonig (1961).

In other experiments leucocidin-treated leucocytes were incubated in 2 ml. of calcium-free Hanks solution for 10 min. at 37°, and then 0·02 ml. of 0·11 M-$CaCl_2$ and 0·12 ml. of 16 mM-ATP were added and incubation was continued for a further 5 min. The suspensions were cooled to 0°, centrifuged at 200 rev./min. for 3 min. and the supernatants rejected. The cell pellets were fixed with ice-cold 2% (w/v) osmium tetroxide (Caulfield, 1957) for 45 min. and dehydrated with ethanol. They were stained for 1 hr. in ethanolic 1% (w/v) phosphotungistic acid and then embedded in Araldite.

The stained sections were examined and photographed with a Philips electron microscope 100 B at 40 kv.

RESULTS

Extrusion of the granule proteins from leucocytes treated with leucocidin or vitamin A. The effects of calcium and ATP on the extrusion of the granule proteins were assessed from the appearance of β-glucuronidase in the supernatant of the leucocidin-treated cell (Woodin & Wieneke, 1963*b*). The release of ribonuclease and peroxidase is also dependent on the presence of calcium and ATP (Table 1). These enzymes are known to be present in the cytoplasmic granules (Cohn & Hirsch, 1960; Woodin, 1962). In the experiments of Woodin & Wieneke (1963*b*) the effect of ATP was observed by adding it to leucocytes before adding leucocidin. Table 1 shows that the stimulated extrusion of the granule proteins is also found when ATP is added after incubation in a calcium-free medium.

Plate 1 (*e*) shows a leucocidin-treated leucocyte in which the extrusion of proteins has been induced by adding calcium and ATP after 10 min. of incubation in calcium-free Hanks solution. In three places, pieces of membrane project from the cell surface membrane. These could be regions where granules have fused with the cell surface and discharged their contents to the cell exterior. The cells

Table 1. *Extrusion of granule enzymes from leucocytes treated with leucocidin and vitamin A*

Leucocytes in calcium-free Hanks solution containing EDTA (magnesium salt) were incubated with leucocidin at 37° or with vitamin A at 0° for 10 min. Calcium and ATP were then added and the suspensions incubated at 37° for 10 min. The enzyme contents of the cell supernatant were determined.

	Enzyme in cell supernatant (units/10^8 cells)					
	Cells treated with leucocidin			Cells treated with vitamin A		
Additions	β-Glucuronidase	Peroxidase	Ribonuclease	β-Glucuronidase	Peroxidase	Ribonuclease
None	7·8	0·14	9·0	16	0·6	17
ATP (1·6 μmoles/ml.)	4·2	0·11	4·4	5·7	0·3	11
Calcium (1 μmole/ml.)	32	0·54	38	46	1·8	54
Calcium (1 μmole/ml.) + ATP (1·6 μmoles/ml.)	81	4·2	110	86	3·4	99
Calcium (1 μmole/ml.) + ATP (0·8 μmole/ml.)	74	3·2	84	69	2·8	84

in Plate 1 (*e*) resemble leucocidin-treated leucocytes prepared by incubation in Hanks medium containing calcium (Plate 1*c* of Woodin *et al.* 1963). The precipitate of extracellular protein present in the preparation shown by Woodin *et al.* (1963) is absent from that shown in Plate 1 (*e*) of the present work as in this case the cells were separated from the supernatant before fixation.

It was suggested that leucocidin does not play a direct part in the extrusion of protein from the cell (Woodin & Wieneke, 1963*b*). Support for this is provided by the finding that after treatment of leucocytes with vitamin A the extrusion of the granule proteins can occur. Incubation of leucocytes (5×10^7/ml. in Hanks medium) at 37° for 10 min. with 300 μg. of vitamin A/ml. induced a maximal release of β-glucuronidase, peroxidase and ribonuclease. Under these conditions the addition of less than 250 μg. of vitamin A/ml. did not induce release of the granule proteins from the cell. The centrifuged pellets of cells treated with vitamin A were yellow, suggesting that adsorption of the vitamin occurs and is accompanied by oxidation of the vitamin (cf. Dingle & Lucy, 1963). The omission of calcium diminished the amount of β-glucuronidase extruded from vitamin A-treated leucocytes. The dependence of the extrusion process on the presence of calcium and ATP was shown by treating leucocytes with vitamin A and EDTA (magnesium salt) at 0° for 10 min. and then incubating the suspensions at 37°. Extrusion of granule proteins occurred only when calcium was present, and it was greatly stimulated by ATP (Table 1).

Effect of the medium on the extrusion of the granule proteins from the leucocidin-treated leucocyte. The optimum concentration of calcium in the extrusion process is about 0·1 mM when it is added to cells at the same time as leucocidin (Woodin & Wieneke, 1963*b*). Very high concentrations of calcium inhibit extrusion (Fig. 1*a*). The effect is more pronounced when calcium is added after incubating the

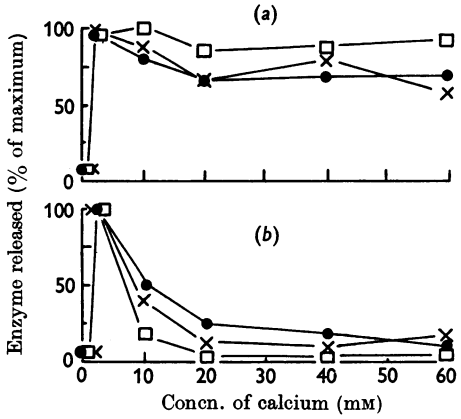


Fig. 1. Inhibition by high calcium concentration of the extrusion of granule proteins. (*a*) ATP (1·65 μmoles/ml.) and calcium were added before leucocidin, and incubation was continued for 10 min. (*b*) Leucocytes were treated with leucocidin for 10 min., and ATP (1·65 μmoles/ml.) and calcium were then added; incubation was continued for 10 min. In both experiments the cells were suspended in calcium-free phosphate-free Krebs–Ringer bicarbonate solution. The enzymes present in the cell supernatant were determined: ●, β-glucuronidase; ×, ribonuclease; □, peroxidase. Maximum activities (units/10^8 cells) were: (*a*) β-glucuronidase, 113; ribonuclease, 59; peroxidase, 10. (*b*) β-Glucuronidase, 85; ribonuclease, 51; peroxidase, 8.

leucocidin-treated cells in calcium-free media (Fig. 1*b*). Under these conditions a small increase in calcium concentration can produce a very large inhibition of the extrusion of proteins. It is possible that the variations in the amount of protein extruded under these conditions are due to this (Table 5 of Woodin & Wieneke, 1963*b*). Fig. 2 shows that the extrusion of β-glucuronidase is inhibited by high magnesium concentrations and that the extent of the inhibition is dependent on

the calcium concentration. The inhibitory action of magnesium was not decreased by adding ATP (10 mM). Fig. 3 shows that inhibition of the extrusion of β-glucuronidase and peroxidase occurs at high ATP concentrations. The ATP concentration at which maximum extrusion occurs is dependent on the calcium concentration. Thus the concentration of ATP giving maximal extrusion is very restricted, and differences in the ATP content of the leucocidin-treated cell can account for some of the variations recorded previously (Table 5 of Woodin & Wieneke, 1963*b*).

Table 2 shows that considerable alterations can be made in the medium without affecting the amount of β-glucuronidase or ribonuclease extruded from the leucocidin-treated cell. Extrusion of peroxidase does not occur in sucrose solution, but other changes have little effect. Fig. 4 shows that replacement of the high-sodium medium by a high-potassium medium does not affect the efficiency of suboptimum concentrations of calcium.

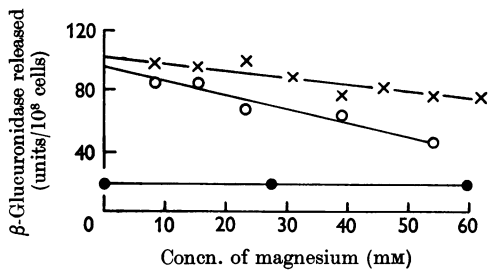


Fig. 2. Inhibition by magnesium of the extrusion of β-glucuronidase. Leucocytes in Hanks medium, with the magnesium and calcium contents indicated, were incubated with leucocidin for 10 min., and the β-glucuronidase in the cell supernatant was determined. Concn. of calcium: ×, 1·65 mM; ○, 0·165 mM; ●, 0.

There does not appear to be competition between calcium and potassium in the extrusion process.

The following compounds did not inhibit the extrusion of β-glucuronidase induced by calcium and stimulated by ATP (final concentrations of the reagents are given): vitamin E, 0·27 mM; carbon

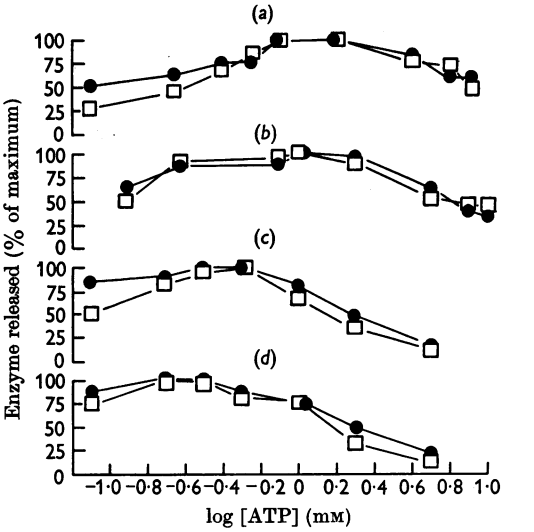


Fig. 3. Inhibition by high concentration of ATP of the extrusion of granule proteins. Leucocidin-treated leucocytes were incubated in calcium-free Hanks medium containing EDTA (magnesium salt) for 10 min. Calcium and ATP were then added and incubation was continued for 10 min. The β-glucuronidase (●) and peroxidase (□) in the cell supernatant were determined. Concn. of calcium: (*a*) 1·42 mM; (*b*) 0·27 mM; (*c*) 0·19 mM; (*d*) 0·11 mM. Maximum activities (units/10⁸ cells) were: (*a*) β-Glucuronidase, 91; peroxidase, 7·8. (*b*) β-Glucuronidase, 94; peroxidase, 8·4. (*c*) β-Glucuronidase, 82; peroxidase, 7·5. (*d*) β-Glucuronidase, 82; peroxidase, 8.

Table 2. *Effect of the medium on the extrusion of the granule proteins from leucocidin-treated leucocytes*

Leucocytes in calcium-free Hanks medium containing EDTA (magnesium salt) were incubated with leucocidin for 10 min. and centrifuged. The cells were suspended in the media described below and incubated for a further 10 min. The enzyme contents of the cell supernatants were determined.

Medium	Enzyme in cell supernatant (units/10⁸ cells) β-Glucuronidase	Ribonuclease	Peroxidase
0·32 M-Sucrose	19	14	0·03
0·32 M-Sucrose–1·25 mM-calcium	34	46	0·04
0·32 M-Sucrose–1·25 mM-calcium–1·65 mM-ATP	87	110	0·05
Hanks medium (calcium-free)	12	18	0·07
Hanks medium–1·25 mM-calcium	45	47	0·45
Hanks medium–1·25 mM-calcium–1·65 mM-ATP	72	99	4·0
0·165 M-NaCl	16	28	0·09
0·165 M-NaCl–1·25 mM-calcium	49	59	0·72
0·165 M-NaCl–1·25 mM-calcium–1·65 mM-ATP	63	82	4·3
0·165 M-KCl	22	24	0·07
0·165 M-KCl–1·25 mM-calcium	54	85	0·16
0·165 M-KCl–1·25 mM-calcium–1·25 mM-ATP	66	100	3·1

tetrachloride, 3·5 mM; cholesterol, 0·5 mM; puromycin, 0·42 mM; ethionine, 1·65 mM; ethanol, 170 mM; sodium azide, 15·4 mM; sodium Amytal, 1·0 mM; Menapthone sodium bisulphite, 0·75 mM; chlorpromazine hydrochloride, 0·3 mM; promethazine hydrochloride, 0·33 mM (vitamin E, carbon tetrachloride and cholesterol were added as ethanolic solutions; other substances were added as aqueous solutions).

Participation of adenosine triphosphate in the extrusion process. Woodin & Wieneke (1963*b*) found that ATP decreased the leakage of aldolase from the leucocidin-treated leucocyte and suggested that ATP contributed to the structure of the cell surface. Direct evidence for the adsorption of ATP by the cell is provided by the uptake of [^{32}P]ATP by leucocidin-treated leucocytes at 0°. This uptake could be due to penetration into the cell water, adsorption on the cell surface, or both. That adsorption on the surface takes place is indicated by the failure of increasing amounts of ATP in the supernatant to produce a corresponding increase in the concentration in the cell water (Fig. 5).

The experiments of Woodin & Wieneke (1963*b*) showed that, when ATP was present, incubation in a calcium-free medium did not decrease the amount of β-glucuronidase released when calcium was subsequently added. The present experiments indicate that it is immaterial if ATP is added before or after the incubation in calcium-free media. Thus ATP may be effective not as a preservative but as a participant in the extrusion process, and the stimulated extrusion that it induces can be due to replacement of cellular ATP, depleted during the incubation in the absence of calcium. There is little stimulation of the extrusion of β-glucuronidase by adenosine or AMP (Woodin & Wieneke, 1963*b*), and it appears that the stimulation is a function of the terminal phosphate groups. Table 3 shows that, after stimulation by [^{32}P]ATP of the extrusion of β-glucuronidase, 90% of the added radioactivity can be recovered and nearly all of this is found in the acid-soluble fraction. There is no evidence that exchange of phosphate groups between ATP and a structural component of the cell occurs during the extrusion of protein.

Confirmation of the absence of a phospholipid–ATP exchange during the extrusion of proteins was obtained from the absence of an increased specific radioactivity in the phospholipid fraction of the cell during the extrusion of proteins (Table 4).

Accumulation of calcium and orthophosphate in the granule fraction of the leucocyte. Table 5 shows that calcium and orthophosphate are present in the granules and the vesicles, and the results are consistent with there being more calcium in the vesicles than in the granules. When granules were incubated at 37° with calcium or with calcium and ATP there was no accumulation of calcium. Less than 10% of the calcium was extracted from the

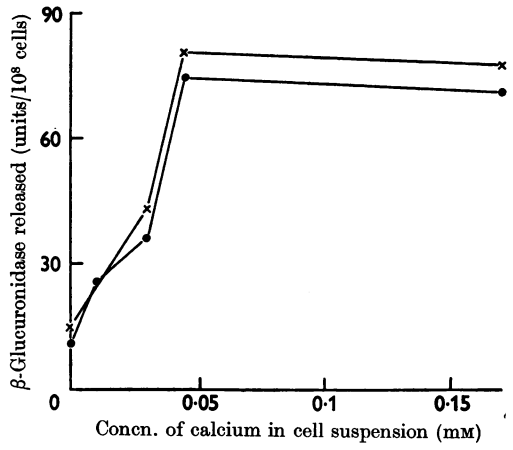


Fig. 4. Effect of potassium on the extrusion of β-glucuronidase induced by calcium. Leucocidin-treated leucocytes were incubated for 10 min. in calcium-free Hanks medium containing EDTA (magnesium salt) and then transferred to solutions of NaCl or KCl containing ATP (1·65 mM). Calcium was added and incubation continued for a further 10 min. The β-glucuronidase in the cell supernatant was determined. ●, 0·165 M-NaCl; ×, 0·165 M-KCl.

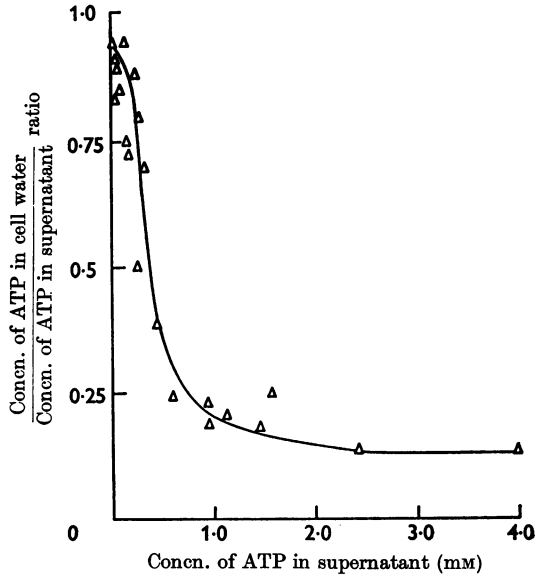


Fig. 5. Uptake of [^{32}P]ATP by leucocidin-treated leucocytes. Suspensions of leucocytes were incubated with leucocidin in calcium-free Hanks medium for 10 min. and then cooled to 0°. [^{32}P]ATP and calcium were added and the suspensions maintained at 0° for 10 min. The concentrations of [^{32}P]ATP in the supernatant and in the cell water were determined.

Table 3. *Distribution of radioactivity in the leucocyte after the extrusion of β-glucuronidase stimulated with adenosine [^{32}P]triphosphate*

Leucocytes in calcium-free Hanks medium containing EDTA (magnesium salt) were incubated with leucocidin for 15 min., and then [^{32}P]ATP and calcium added to some samples. Incubation was continued for a further 8 min. and the β-glucuronidase in a sample of the cell supernatants determined. The remainder of the cell suspension was precipitated with $HClO_4$ and the total radioactivity in the cell fractions determined.

Additions	β-Glucuronidase in cell supernatant (units 10^8/cells)	Radioactivity recovered (% of added radioactivity)				
		Ortho-phosphate	Acid-soluble esterified phosphate	Lipid phosphorus	Residual acid-insoluble phosphorus	Total
None	16	—	—	—	—	—
[^{32}P]ATP (1·6 μmoles/ml.)	16	35	54	<1	<1	89
Calcium (1·6 μmoles/ml.)	74	—	—	—	—	—
[^{32}P]ATP (1·6 μmoles/ml.) + calcium (1·6 μmoles/ml.)	130	28	68	<1	<1	94

Table 4. *Specific radioactivity of the lipids of leucocytes after the extrusion of β-glucuronidase stimulated with adenosine [^{32}P]triphosphate*

Leucocytes were incubated with leucocidin in calcium-free Hanks medium for 10 min. and then [^{32}P]ATP or calcium was added to some samples. Incubation was continued for a further 10 min. and the β-glucuronidase in the cell supernatant determined. The cell pellets were cooled to 0° and [^{32}P]ATP was added to a cell pellet that had been incubated with calcium only. The specific and total radioactivities of the lipids in the cell pellets were determined.

Additions	β-Glucuronidase in cell supernatant (units/10^8 cells)	Specific activity of lipids (counts/min./100 μg. of phosphorus)	Recovered radioactivity (% of added radioactivity)
None	13	—	—
[^{32}P]ATP (1·6 μmoles/ml.)	13	1·48	0·09
Calcium (1·6 μmoles/ml.); [^{32}P]ATP (1·6 μmoles/ml.) added at 0°, after incubation	45	0·56	0·045
[^{32}P]ATP (1·6 μmoles/ml.) + calcium (1·6 μmoles/ml.)	130	0·74	0·045

Table 5. *Calcium content and orthophosphate content of the granule fraction of the leucocyte*

The granules were isolated from normal cells washed with calcium-free Hanks solution. The vesicles were isolated from leucocidin-treated leucocytes incubated in Hanks solution containing calcium (1·65 mM). The analyses were made on extracts of the pellets of granules or vesicles in 5% (w/v) trichloroacetic acid.

	Calcium content (μmole/unit)	Orthophosphate content (μmole/unit)
Vesicles	0·185	0·056
Granules	0·020	0·010
Granules incubated with 0·32 M-sucrose–1·0 mM-calcium, pH 7·5	0·014	0·004
Granules incubated with 0·32 M-sucrose–1·0 mM-ATP–1·0 mM-calcium, pH 7·5	0·014	0·012

granules or the vesicles by water adjusted to pH 10 with 5 N-ammonia or by chloroform–methanol (2:1, v/v). More than 90% of the calcium and orthophosphate was extracted with 5% (w/v) trichloroacetic acid.

After lead-staining, the granules in normal cells and those in cells treated with leucocidin in the absence of calcium appear empty (Plate 1*a* and 1*b*). The granules of cells treated with leucocidin in the presence of calcium contain a very dense material after lead-staining (Plate 1*c*). This is absent from sections extracted with citric acid before lead-staining (Plate 1*d*).

Release of protein from isolated granules. The stability of preparations of the granules differed considerably. Some preparations liberated 30% of their β-glucuronidase on incubation at 25° for 15 min. As the granules remain intact in the leucocidin-treated cell incubated in the absence of calcium at 37° for at least 20 min. there are conditions in the intact cell that increase the stability of the granules. The addition of 100 μg. of EDTA (magnesium salt)/ml. to the sucrose used in the preparation of the granules did not increase their stability.

Table 6 gives the effects of different media on the retention of protein by a sample of granules that was fairly stable in sucrose solution. Under all conditions peroxidase is retained, and the retention

Table 6. *Release of some enzymes from isolated granules of the leucocyte*

Granules were incubated in the media indicated below at 37° or maintained at 0° for 10 min. and centrifuged. The enzyme contents and total protein content of the supernatant were determined. The total amount of the enzymes in the granules is the amount found in a suspension of granules in water which had been frozen and thawed six times.

Medium	Incubation temperature	Amount in cell supernatant (% of total amount in granules) β-Glucuronidase	Peroxidase	Ribonuclease	Total protein
0·32 M-Sucrose	0°	3	1	2	2
	37	3	1·8	6	8
Hanks medium (calcium-free) + 100 μg. of EDTA (magnesium salt)/ml.	0	9	2·4	9	14
	37	11	5	14	36
Hanks medium–1·0 mM-ATP–1·0 mM-calcium	0	9·6	3·2	10	15
	37	9	4·2	20	45
Hanks medium + 200 μg. of vitamin A/ml.	0	11	2·5	12	29
	37	24	3·2	11	37
Hanks medium diluted 20-fold with water	0	—	2·7	56	90
	37	—	5·0	61	90
Hanks medium diluted 20-fold with water–1·0 mM-ATP–1·0 mM-calcium	0	70	3·2	56	90
	37	75	6·2	61	90

of β-glucuronidase or ribonuclease is not affected by calcium or ATP. Thus the release of these enzymes from isolated granules differs considerably from the release from the intact cell.

Properties of the adenosine triphosphatase of the leucocyte granules. The hydrolysis of ATP and GTP by the granules isolated from normal leucocytes was reported by Woodin & Wieneke (1963*b*). Under the conditions used in the present work the release of orthophosphate from ATP was directly dependent on the granule concentration when that was less than 12 units/ml. Some other properties are recorded in Fig. 6. With ATP (0·67 μmole/ml.) as substrate, maximum activity could be obtained with 0·67 mM-calcium or -magnesium as activator, and concentrations of these cations less than 3·0 mM were not inhibitory (Fig. 6*a*). With 0·67 mM-magnesium or -calcium, activity was not changed by sodium or potassium concentrations in the range 0–0·2 M (Fig. 6*b*). In the presence of 0·67 mM-magnesium or -calcium, increasing amounts of orthophosphate were liberated as the ATP concentration was increased to 2·0 mM, above which ATP had an inhibitory effect (Fig. 6*c*). The pH optimum was in the range pH 7·8–8·2 (Fig. 6*d*).

The adenosine-triphosphatase activity of the granules of the leucocyte is tightly bound. After freezing and thawing six times no activity could be found in the granule supernatant. Freezing and thawing in this way did not increase the total adenosine-triphosphatase activity of the granule suspension, suggesting that the enzyme is freely accessible to substrate in the normal granule.

The properties of the adenosine triphosphatase of the leucocyte granules distinguish it from the enzyme of the microsomes and cell surface (Deul & McIlwain, 1961). The failure of dinitrophenol to stimulate ATP breakdown by the granules (Woodin & Wieneke, 1963*b*) indicates that the activity is not due to mitochondrial contamination. It is not clear if an adenosine triphosphatase with properties identical with those of the leucocyte granules is present in the granules of other secretory tissues, but Danielli (1952) has described the histochemical properties of an alkaline phosphatase in secreting tissues.

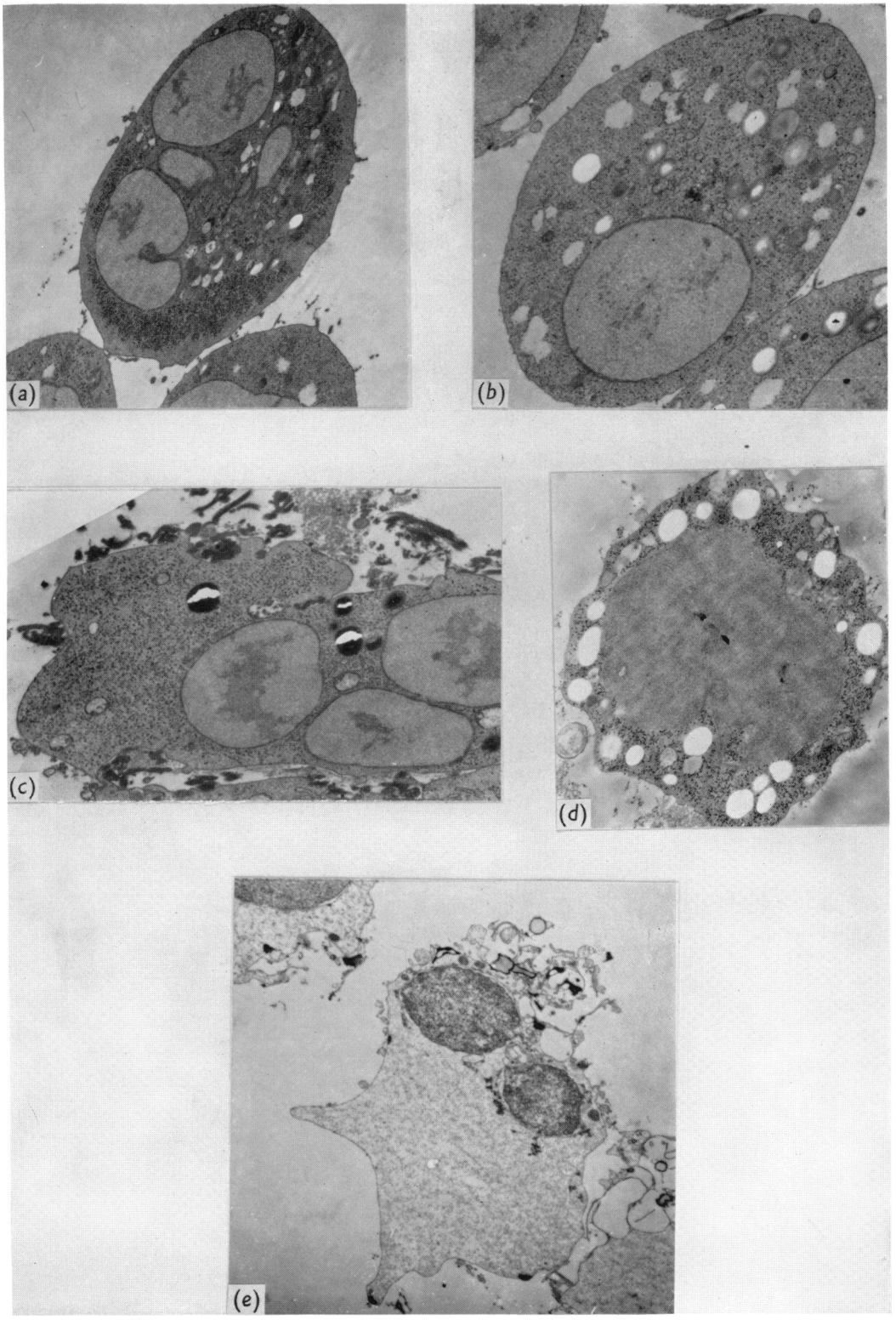
DISCUSSION

The results presented above provide further evidence that the extrusion of granule protein from the leucocyte proceeds through a complex interaction of the membranes of the cell surface and the granule and not by intracellular lysis of the granule followed by diffusion of the liberated proteins through the cell surface membrane. The release of protein from isolated granules is not affected by calcium, ATP or EDTA, reagents that modify the release of the granule proteins from the intact leucocidin-treated leucocyte.

The granule proteins liberated from the leucocidin-treated leucocyte are a very heterogeneous

EXPLANATION OF PLATE 1

(*a*) Normal leucocytes. (*b*) Leucocytes treated with leucocidin in the absence of calcium. (*c*) Leucocytes treated with leucocidin in the presence of calcium. (*d*) Leucocytes treated with leucocidin in the presence of calcium. In all cases the cells were fixed with permanganate and stained with lead. The section of the cells in (*d*) was extracted with citric acid before staining with lead. (*e*) Leucocytes incubated with leucocidin in calcium-free Hanks solution and then with calcium and ATP; the cells were fixed with osmium and stained with phosphotungstic acid.


(a)
(b)
(c)
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(e)

collection. At least five electrophoretic components can be distinguished (Woodin, 1961), and the sedimentation boundary is very asymmetric

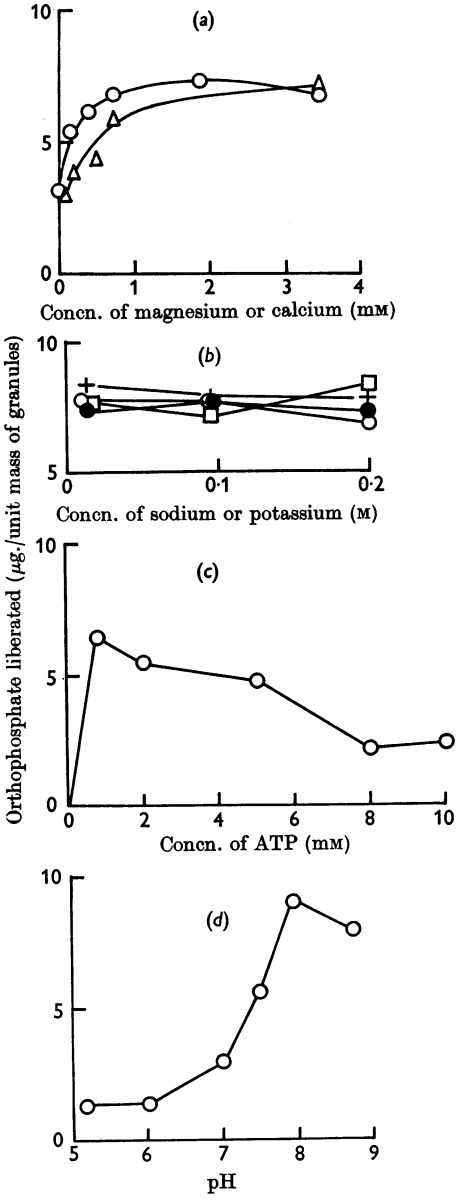

Fig. 6. Adenosine-triphosphatase activity of leucocyte granules. (*a*) The effect of calcium or magnesium: △, calcium present; ○, magnesium present. (*b*) The effect of sodium and potassium: □, sodium and magnesium present; +, sodium and calcium present; ●, potassium and magnesium present; ○, potassium and calcium present. (*c*) The effect of ATP concentration. (*d*) The effect of pH. In all cases granules suspended in sucrose were incubated for 15 min. at 37°.

(Fig. 3*c* of Woodin, 1961). Similarly, the granules of the leucocyte are heterogeneous: three morphological forms can be seen (Florey & Grant, 1961). It is possible that there are different modes of combination of the proteins in the intact granule, and the conditions under which they pass into solution may be different. It is not to be expected that all proteins will be released under all conditions, and in the present work differences in the conditions of release of peroxidase and β-glucuronidase have been observed. This is not taken as evidence that the mechanism of extrusion of these proteins differs from that suggested by Woodin *et al.* (1963), that is by fusion of the granule with the cell surface, dissolution of the granule contents and passage of the proteins through the cell surface membrane, which may be broken.

Woodin & Wieneke (1963*b*) showed that leucocidin does not play a direct part in the extrusion process, and the present paper shows that treatment of leucocytes with vitamin A can also induce the extrusion of the granule proteins. The degranulation that follows phagocytosis proceeds by fusion of the granule with the membrane of the phagocytosis vesicle (Hirsch, 1962). It is thus possible that the condition of the cell membrane that permits discharge of the granule contents is common to that treated with leucocidin or vitamin A or 'internalized' in the cytoplasm as the membrane of a phagocytosis vesicle. Alternatively, no change in the membrane may be required and the action of these agents may be to permit collision of the granule and the cell surface membrane.

Both calcium and ATP are necessary for release of the granule contents, but both, in high concentrations, can inhibit the process. This finding, and the complex interrelationship of the concentrations of calcium and ATP giving a maximum effect, suggest that, though both reagents are necessary for extrusion to occur, both must be removed for its completion. It may be that, when calcium and ATP are present, sites are produced in the membrane leading to adherence of the granule and that then a reaction occurs which eliminates both calcium and ATP from the site of contact. Calcium gives mechanical rigidity to the cell surface, and both calcium and ATP decrease the permeability of the cell membrane (Woodin & Wieneke, 1963*b*), so that their removal from the site of contact will produce a local region of increased permeability and decreased mechanical strength. These conditions will be conducive to the release of the proteins of the granule by diffusion through the membrane or after its mechanical rupture.

These considerations are also relevant to the problem of the fusion of the membranes of the granule and the cell surface. This would be expected to occur when the molecular interactions

across the gap between the membranes became as strong as those within the membranes. The removal of calcium and ATP at the site of fusion, with the consequent decrease in cross-linking, followed by random movements of the molecules in the membrane, would contribute to this condition.

A sequence of reactions that would lead to removal of calcium and ATP and to fusion might be initiated by hydrolysis of the ATP in the cell membrane by the adenosine triphosphatase of the granules. This would give a localized high orthophosphate concentration and competition for the binding of calcium would be expected. Glycolysis is inhibited in the leucocidin-treated cell, and the accumulation of orthophosphate could be considerable. The cell is permeable to calcium, and the solubility product of calcium phosphate could be exceeded and deposition of the solid phase might occur. The present work shows that the vesicles of the leucocidin-treated cell are rich in calcium and orthophosphate, and electron micrographs show the deposition of a material that could contain calcium phosphate. Woodin *et al.* (1963) consider that some of the vesicles of the leucocidin-treated cell are derived from granules that have discharged part of their contents into the medium.

It is known that calcium can change the physical state of fatty acid monolayers (Archer & La Mer, 1955), and it is also known that calcium can penetrate a phospholipid monolayer and that this is modified by ATP (Kimizuka & Koketsu, 1962). There is no direct evidence on the nature of the molecules in the membrane that bind calcium, but there is circumstancial evidence that the phospholipids are involved. When calcium is absent the incorporation of ^{32}P into the phospholipids, relative to that in ATP, is stimulated 12-fold (Table 5 of Woodin & Wieneke, 1963*a*). The conversion of membrane phospholipids into a solid state by the binding of calcium would decrease the opportunity for thermal movement and collision with the enzyme catalysing the exchange of phosphate groups.

Previous papers have indicated a parallel between secretion in the leucocidin-treated leucocyte and in the pancreas and adrenal medulla (Woodin, 1962; Woodin & Wieneke, 1963*b*; Woodin *et al.* 1963). Stimulation of secretion in the pancreas and adrenal medulla leads to increased incorporation of radioactivity into the phospholipids (Hokin & Hokin, 1958; Hokin, Benfey & Hokin, 1958), and the characteristics of this process are consistent with this being due to an increased accessibility of the phospholipids to the enzymes catalysing the exchange (Woodin, 1963). This could arise from the elimination of calcium at the site of fusion of the secretory granule and the surface membrane. In the pancreas and adrenal medulla the phosphatidyl inositide and 'phosphatidic acid' fractions became heavily labelled when secretion is stimulated. Though it is possible that these are located at the sites of fusion of the secretory granules and the cell membrane, this interpretation must be treated with caution. These acidic fractions are the most metabolically active phospholipids in the resting tissue and it has not yet been shown that the increased radioactivity is an increased specific activity (Kennedy, 1962; Woodin, 1963).

SUMMARY

1. The extrusion of the granule proteins from the leucocyte can be induced by vitamin A if calcium is present. The process is stimulated by ATP.

2. The extrusion from the leucocidin-treated leucocyte can take place in potassium chloride, sodium chloride or sucrose solution. It is stimulated by low but inhibited by high calcium or ATP concentrations. The optimum ATP concentration is dependent on the calcium concentration. Magnesium is inhibitory.

3. When extrusion is stimulated with [^{32}P]ATP 90 % of the radioactivity can be recovered and all is in the acid-soluble fraction. The specific radioactivity of the phospholipids is not increased.

4. The release of enzymes from the isolated granules is not stimulated by calcium or ATP.

5. Some properties of the adenosine triphosphatase of the granules are described.

6. It is suggested that, though both calcium and ATP must be present for the adherence of the granules to the cell surface membrane, they must both be removed for the extrusion of protein to occur. A mechanism involving hydrolysis of ATP by the adenosine triphosphatase of the granules is described. The fusion of the membranes of the granule and cell surface may be facilitated by decreased cross-linking in the membranes after the elimination of calcium and ATP.

7. The participation of phospholipids in the extrusion process is discussed.

Dr J. E. French kindly provided the electron micrographs in Plate 1 (*a*), 1 (*b*) and 1 (*c*). Mr H. Showell provided able technical assistance.

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The Assay and Reaction Kinetics of Leucine Aminopeptidase from Swine Kidney

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Kinetic investigation of leucine aminopeptidase (EC 3.4.1.1) has been hampered by lack of suitable methods for following the hydrolytic reaction. The method of Grassmann & Heyde (1929) has been widely used, but its lack of precision makes it suitable only for investigating the specificity of the enzyme. For kinetic investigations, it is desirable to have a continuous record of the reaction and a pH-stat assay has been developed for this purpose.

The enzyme is inhibited by alcohols (Hill & Smith, 1957), and it has been suggested that this is due to competition of the alcohol for the site binding the substrate side chain. A low rate of esterase activity has also been reported (Smith & Polglase, 1949; Smith & Spackman, 1955; Shippey & Binkley, 1958) with leucine esters of small aliphatic alcohols as substrates. Owing to the key importance of the esterase activity and inhibition by alcohols for any theory of the mode of action of the enzyme, we have investigated these problems in some detail.

MATERIALS AND METHODS

Water. Glass-distilled water was passed successively through columns of De-Acidite FF, Zeo-Karb 225 and 215 (mixed) and Bio-Deminrolit (The Permutit Co. Ltd., London).

Sodium chloride. This was either Specpure (Johnson Matthey and Co. Ltd., Hatton Garden, London) or recrystallized from EDTA solution.

Hydrochloric acid. AnalaR hydrochloric acid was diluted and distilled. Solutions of the required dilution were standardized potentiometrically against recrystallized tris (Fossum, Markunas & Riddick, 1951).

Sodium hydroxide. AnalaR sodium hydroxide solution (5N) was passed through a column of Zeo-Karb 225 previously equilibrated with AnalaR sodium chloride.

Substrates. L-Leucine amide, L-leucylvaline, L-leucyl alanine, and L-leucine benzyl ester were obtained from the Yeda Research and Development Ltd. (Rehovoth, Israel). L-Leucylglycine was obtained from Roche Products Ltd. (Welwyn Garden City, Herts.). All the compounds were homogeneous by paper chromatography in butanol–acetic acid–water (4:1:5, by vol.).

Glycerol and butanol. The commercial products were redistilled.

N-*Ethylmorpholine.* This was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., and twice redistilled. It was converted into the hydrochloride by passing anhydrous HCl through a solution in ethanol. The solid was recrystallized twice from acetone–ethanol (2:1, v/v).

Enzyme. This was prepared according to the published method (Hill, Spackmann, Brown & Smith, 1958) as far as the acetone precipitate. Chromatography (Visvanatha, 1959) was substituted for starch-column electrophoresis. A linear gradient of sodium chloride in 5 mM-*N*-ethylmorpholine buffer, pH 8·0 or 8·4, made 5 mM with respect to magnesium chloride, was employed. The enzyme emerged as a narrow band at the front of the main protein peak at about 0·1 M-sodium chloride. A typical chromatogram is shown in Fig. 1. The contents of the tubes containing the enzyme were combined and rechromatographed under identical conditions, when the protein and activity emerged as a single peak.