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The Extrusion of Protein from the Rabbit Polymorphonuclear Leucocyte treated with Staphylococcal Leucocidin

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When rabbit polymorphonuclear leucocytes are incubated with staphylococcal leucocidin protein is released from the cell but only small amounts of amino acids, nucleotides and phosphate esters. The protein is not derived from the soluble protein of the cell cytoplasm and it was suggested that it originates from the characteristic granules of the leucocyte (Woodin, 1961). The preparation and composition of the granules of the leucocyte have been described by Cohn & Hirsch (1960*a*) and the present paper shows that many of the enzymes of the granules are present in the supernatant of the leucocidin-treated leucocyte.

The transfer of protein from the granules to the exterior of the cell has features in common with the extrusion phase of protein secretion in the mammalian cell (Junqueira & Hirsch, 1956), and this has prompted a preliminary investigation of the effect of leucocidin on the incorporation of radioactive phosphorus into the phospholipids of the leucocyte.

MATERIALS AND METHODS

Leucocytes. These were taken from the peritoneal cavity of rabbits 5–10 hr. after the injection of 400 ml. of 0·85% NaCl. The exudates were centrifuged at 200*g* for 5 min. and the cells resuspended in a small volume of the supernatant. After a few minutes the leucocytes aggregated and fell to the bottom of the tube, leaving any red cells in the supernatant. The leucocytes were freed from all red-cell contamination in this way and then washed three times with Hanks (1948) medium by centrifuging at 200*g* for 5 min.

Leucocidin. The preparation of the two components of staphylococcal leucocidin was described by Woodin (1960). Each component was added to leucocyte suspensions to give 2 μg./10^8 cells.

Chemical methods. These were described by Woodin (1961).

Preparation of cell homogenates. Suspensions of leucocytes (10 ml. containing 10^9 cells in Hanks medium) were incubated at 37° alone or with added leucocidin and then centrifuged for 5 min. at 500*g* and the cell supernatants removed. The preparation of the subcellular fractions from the cell pellets was carried out essentially as described by Cohn & Hirsch (1960*a*). Complete cell breakage was not obtained by the initial rapid pipetting (see Cohn & Hirsch, 1960*a*) and after removal of the supernatant from the partially lysed cells these were homogenized in 0·34 M-sucrose in a Potter–Elvehjem tube until 100% cell breakage was obtained. The separation of the 'nuclear', 'granule' and 'post-granule' fractions was done as described by Cohn & Hirsch (1960*a*). The post-granule fraction is the supernatant obtained after removal of the granules by centrifuging at 8200*g* for 30 min. It was centrifuged at 110 000*g* for 45 min. and the supernatant separated from the sediment, which was then suspended in 0·34 M-sucrose. The latter fractions will be called the soluble fraction and the post-granule sediment respectively.

Estimation of enzyme activities. The assay and the units of activity of acid phosphatase, alkaline phosphatase, ribonuclease, deoxyribonuclease and β-glucuronidase were those used by Cohn & Hirsch (1960*a*).

Peroxidase activity was determined by a modified purpurgallin method. Tubes were set up with 3 ml. of 0·8% pyrogallol, 1 ml. of 0·25 M-sodium phosphate buffer, pH 6·5, and 0·5 ml. of enzyme solution. Then 0·5 ml. of 15 mM-H_2O_2 was added, the contents of the tubes were mixed and incubated at room temperature for 10 min. and the reaction was stopped by addition of 0·2 ml. of 2 N-H_2SO_4 and 1 ml. of amyl alcohol. The tubes were shaken and centrifuged. All the yellow colour was transferred to the amyl alcohol layer and determined by measuring the extinction at 430 mμ. The unit of activity is defined as the amount of enzyme producing an increase in extinction at 430 mμ of 1·0 under these conditions.

Cytochrome *c*-oxidase activity was estimated by the method of Cooperstein & Lazarow (1951), succinoxidase activity was measured by the method of Schneider & Potter (1943) and succinic-dehydrogenase activity was estimated by ferricyanide reduction (Whittaker, 1959). Oxidation of reduced diphosphopyridine nucleotide was observed by measuring changes in extinction at 340 mμ in 0·05 M-sodium phosphate buffer, pH 7·5.

In all cases material derived from the leucocyte was frozen and thawed six times before assay for the above-mentioned activities.

Estimation of nucleic acids. The procedure for this was described by Woodin (1961).

Preparation of radioactive lipids from the leucocyte. Duplicate suspensions (20 ml. containing 10^9–10^{10} cells) were incubated at 37° for 20 min. in Hanks medium containing ^{32}P, 2×10^4–2×10^5 counts/min./ml. To one suspension of cells leucocidin was then added and incubation continued for a further 10 min. To test the efficiency of removal of non-lipid contaminants the cell suspensions were then treated in one of three different ways.

Procedure *A*. The suspensions were diluted to 250 ml. with ice-cold Hanks medium and centrifuged at 500*g* for 5 min. The cell pellets were washed once in ice-cold Hanks

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medium and were then extracted with 20 vol. of chloroform–methanol (2:1, v/v), at room temperature for 2 hr. The suspensions were then filtered. To the clear filtrate 0·2 vol. of NaH_2PO_4 (0·2%) was added and the mixture shaken and centrifuged. The upper phase was rejected and the lower phase washed three times with 0·2 vol. of chloroform–methanol–0·2% NaH_2PO_4 (3:48:47, by vol.) and then twice with chloroform–methanol–0·9% NaCl (3:48:47, by vol.) (cf. Folch, Lees & Sloane-Stanley, 1957). The final lower phase was evaporated to dryness and dissolved in dry chloroform. Samples of the chloroform solution were taken for determination of total phosphorus and radioactivity.

Procedure *B*. An extract in chloroform–methanol (2:1, v/v) was prepared as in procedure *A* and shaken with 0·2 vol. of 0·04% $CaCl_2$ and the upper phase rejected. The lower phase was dried and dissolved in chloroform that had been saturated with water. The solution was added to a column of 10 g. of cellulose (Whatman cellulose powder, standard grade) suspended in wet chloroform. The column was eluted with 300 ml. of wet chloroform and the eluate dried. The solid residue was extracted with 20 ml. of dry ether and the insoluble fraction filtered off. The ether solution was washed three times with 0·1 N-HCl–0·1% NaH_2PO_4 and then twice with 0·1 N-HCl and twice with water. Ether was added between each wash to keep the volume of the ether phase constant. Samples of the final ether solution were taken for the determination of total phosphorus and radioactivity.

Procedure *C*. After incubation the cells were cooled in ice and 0·1 vol. of 100% (w/v) trichloroacetic acid was added. The trichloroacetic acid-insoluble fraction was washed three times with 20 vol. of 7% (w/v) trichloroacetic acid containing 0·1% of NaH_2PO_4 and then twice with 20 vol. of 7% (w/v) trichloroacetic acid. The trichloroacetic acid-insoluble fraction was extracted with 20 vol. of chloroform–methanol (2:1, v/v) at room temperature for 2 hr. The extract was filtered, the filtrate dried and the solids were extracted in 20 ml. of ether and the insoluble material was filtered off. The ether solution was then washed three times with 0·1 N-HCl–0·1% NaH_2PO_4, twice with 0·1 N-HCl and twice with water, ether being added between each wash to keep the volume of the ether phase constant. Samples of the final ether solution were analysed for total phosphorus and radioactivity.

Fractionation of the lipids on columns of silicic acid. A sample of radioactive lipids from 10^{10} normal cells and a further sample from 10^{10} leucocidin-treated cells was prepared and washed by procedure *A* (see above). Each was fractionated on silicic acid as described by Woodin (1961), and the five main fractions were prepared (bands A–E, see Fig. 2, Woodin, 1961). During elution with chloroform–methanol (9:1, v/v), 2 ml. fractions were collected until band A had emerged and then a further 150 ml. was collected as a single fraction. The column was then eluted with chloroform–methanol (2:3, v/v) and fractions (2 ml.) were collected until band C had emerged. Then two further fractions of eluate were collected: one, 50 ml. of chloroform–methanol (2:3, v/v), and one of 50 ml. of methanol. The five fractions obtained in this way were concentrated and samples taken for determination of radioactivity and total phosphorus.

Partition of phospholipids between chloroform and methanol–water phases. A sample of lipid prepared by procedure *B* (see above) was dissolved in 20 ml. of chloroform–methanol (2:1, v/v) and shaken with 0·2 vol. of 0·2% Na_2HPO_4. The lower phase was then shaken with five successive portions of 10 vol. of chloroform–methanol–0·2% Na_2HPO_4 (3:48:47, by vol.), the two phases being separated by centrifuging for 20 min. at 3000*g*. Samples of the lower phase were then taken for determination of total phosphorus and radioactivity. The combined upper phases, which had only a trace of opalescence, were concentrated to about 15 ml. and cooled in ice; HCl was then added to give pH 1 and the solution extracted with five portions of 30 ml. of ether. The combined ether extracts were washed twice with 5 ml. of water to remove dissolved HCl, and dried. The product will be called 'upper-phase lipid'. After solution in a small volume of dry ether, samples were taken for determination of total phosphorus and radioactivity. Further samples were used for chromatography on silicic acid-impregnated paper and on Dowex 1 (formate form).

Chromatography on silicic acid-impregnated paper. This was done on Whatman no. 3 paper impregnated with silicic acid by the method of Lea, Rhodes & Stoll (1955). A sample of upper-phase lipid containing 50 μg. of phosphorus was added to the paper, which was then developed with di*iso*butyl ketone–acetic acid–water (40:30:7, by vol.) for 20 hr. The paper was then dried at room temperature and the radioactivity in 1 cm.^{2} areas was determined with a mica end-window Geiger counter. The areas of the paper containing radioactivity were cut out and eluted with chloroform–methanol–water (75:25:2, by vol.). Samples of the eluate were used for determination of radioactivity and total phosphorus.

Chromatography on Dowex 1. This was done by the method of Kennedy (1953). A sample of upper-phase lipid (92 μg. of phosphorus, 800 counts/min.) was dissolved in 10 ml. of 95% (v/v) ethanol and passed down a column (5 cm. × 1·5 cm.) of Dowex 1 (formate form; × 8). The column was washed with 50 ml. of 95% (v/v) ethanol; the effluent was collected, concentrated to a small volume and the total radioactivity determined. The column was then eluted with 150 ml. of 0·1 N-HCl in 95% (v/v) ethanol and the total radioactivity of this fraction determined.

Evaporation of solutions of lipids. This was done in a rotary evaporator. When water remained after evaporation of the organic solvent this was removed by freeze-drying.

Incorporation of ^{32}P into the ribonucleic acids of the leucocyte. Duplicate suspensions of leucocytes (20 ml. containing 4×10^9 cells) were incubated in Hanks medium containing ^{32}P (230 000 counts/min./ml.) for 20 min. at 37°, and then to one suspension leucocidin was added and incubation of both suspensions continued for a further 20 min. The suspensions were diluted to 250 ml. with ice-cold Hanks medium and centrifuged at 500*g* for 5 min. The cell pellets were then washed once with ice-cold Hanks medium and extracted for 2 hr. at room temperature in 20 vol. of chloroform–methanol (2:1, v/v). The insoluble material was recovered by centrifuging and washed once with ether and dried. It was washed three times with 10 vol. of 7% (w/v) trichloroacetic acid–0·1% NaH_2PO_4, three times with 10 vol. of 7% (w/v) trichloroacetic acid, three times with 10 vol. of acetone and dried. The ribonucleotides in this material were prepared, separated and identified by the method described by Davidson & Smellie (1952). Samples of the final solutions were taken for the determination of total phosphorus and radioactivity.

Radioactivity. Samples of solutions of known phosphorus content were dried on planchets and counted with a mica end-window Geiger counter. Carrier-free [^{32}P]orthophosphate was obtained from The Radiochemical Centre (Amersham, Bucks.) and diluted in Hanks medium. The specific activities reported in this paper are corrected to an activity of 10^5 counts/min./ml. of cell suspension. The counting was done at infinite thinness and the surface area of the planchets was 3·9 cm.^{2}

RESULTS

Effect of leucocidin on the composition of the granules

Distribution of protein and nucleic acids in subcellular fractions of normal and leucocidin-treated leucocytes. It was shown that the only nitrogenous non-diffusible materials appearing in the supernatant of leucocidin-treated leucocytes were proteins (Woodin, 1961). In the present work the total trichloroacetic acid-insoluble nitrogen content of the subcellular fractions has been used as a measure of the changes in the protein content induced by leucocidin action. All the deoxyribonucleic acid of normal and leucocidin-treated leucocytes is present in the nuclear fraction, and Table 1 shows that there are no differences in the distribution of ribonucleic acid in the fractions from normal and leucocidin-treated cells.

Table 1 shows that the appearance of protein in the supernatant of leucocidin-treated leucocytes is associated with a decreased protein content of the granular and nuclear fractions of leucocidin-treated cells. Microscopic examination of the nuclear fraction of normal cells showed the presence of many granules, and Cohn & Hirsch (1960*a*) record their inability to free the nuclear fraction completely from granules. Thus the higher protein content of the nuclear fraction from normal cells probably results from its greater content of granules.

Distribution of enzymes in subcellular fractions from normal and leucocidin-treated leucocytes. The presence of lysozyme and phagocytin activities in the supernatant of leucocidin-treated leucocytes was recorded by Woodin (1961). It has now been found that ribonuclease, deoxyribonuclease, β-glucuronidase, peroxidase and small amounts of acid and alkaline phosphatase are also present. No succinoxidase, succinic-dehydrogenase, cytochrome *c*-oxidase or reduced diphosphopyridine nucleotide-oxidase activity could be detected in the supernatant of leucocidin-treated leucocytes.

Table 2 shows that the accumulation of the hydrolytic enzymes in the supernatant of leucocidin-treated leucocytes is paralleled by a decrease in the activity in the granular and nuclear fractions from leucocidin-treated cells. There is only a very small increase in the enzyme activities of the soluble fraction of the leucocidin-treated leucocyte. Thus the release of protein from the granules during leucocidin action is not accompanied by a release of protein into the cytoplasm, which Cohn & Hirsch (1960*b*) have shown to accompany phagocytosis. The total enzymic activity recovered from the leucocidin-treated leucocyte is greater than that recovered from the normal cell, suggesting that the release of the enzymes during leucocidin action is a more efficient method of activation than freezing and thawing.

The data in Table 3 are recorded to indicate the magnitude of the enzyme activities found in some of the leucocyte fractions. Comparison of the specific activities in the cell supernatant with those in the subcellular fractions is complicated by the different methods of activation and the fact that not all the protein of the granules is released to the exterior of the cell (the retention of much of the phosphatases in the granules is recorded in Table 2).

No cytochrome *c*-oxidase activity could be detected in the granule fraction of normal cells, and in the succinic-dehydrogenase test the endogenous activity of the granule fraction was 80% of that observed in the presence of excess of succinate. It is evident that these typical mitochondrial enzymes are in low concentration in the granule fraction.

Table 1. *Distribution of trichloroacetic acid-insoluble nitrogen and ribonucleic acid in fractions derived from normal and leucocidin-treated leucocytes*

Leucocytes were incubated alone and with added leucocidin for 10 min. and the cell supernatant was separated. The cell pellets were then homogenized by sucrose lysis and the subcellular fractions isolated.

	Trichloroacetic acid-insoluble N (μg./10^8 cells)		Ribonucleic acid (μg./10^8 cells)	
Fraction	From normal cells	From leucocidin-treated cells	From normal cells	From leucocidin-treated cells
Cell supernatant	15	260	<2	<2
Soluble fraction of homogenate	160	170	<2	<2
Post-granule sediment	85	85	4·5	4·5
Granule fraction	270	120	17	15
Nuclear fraction	580	460	120	125

No oxidation was observed when the granule fraction of normal cells was added to reduced diphosphopyridine nucleotide either alone or with methylene blue; in this respect the granules of rabbit leucocytes differ from those of the guinea pig (Karnovsky, 1961).

Effect of leucocidin on the incorporation of ^{32}P in the leucocyte

Incorporation of ^{32}P into the phospholipids of the leucocyte. Table 4 shows that the washed lipids of leucocidin-treated leucocytes possess five to six

Table 2. *Distribution of enzymes of the granules in fractions derived from normal and leucocidin-treated leucocytes*

Results are expressed as percentages of the total activity recovered from normal cells.

Fraction	Acid phosphatase	Alkaline phosphatase	Ribonuclease	Deoxy-ribonuclease	β-Glucuronidase	Peroxidase
Leucocidin-treated cell supernatant	15	13	75	87	72	70
Soluble fraction from normal cells	5	3	4	4	8	6
Soluble fraction from leucocidin-treated cells	10	5	3	4	8	6
Granule fraction from normal cells	62	60	58	63	59	30
Granule fraction from leucocidin-treated cells	51	49	13	8	14	7
Nuclear fraction from normal cells	33	37	38	33	33	64
Nuclear fraction from leucocidin-treated cells	32	30	25	18	22	28

Table 3. *Specific activity of some enzymes of the granules in fractions derived from normal and leucocidin-treated cells*

Specific activities are in units/mg. of trichloroacetic acid-insoluble N. For definition of the units see Materials and Methods section.

	Specific activity			
Fraction	Ribonuclease	Deoxyribonuclease	β-Glucuronidase	Peroxidase
Leucocidin-treated cell supernatant	2000	170	0·4	25
Granule fraction from normal cells	1400	110	0·27	18

Table 4. *Specific activity of lipids from normal and leucocidin-treated cells*

Full details of the washing procedures are given in the Materials and Methods section.

		Lipids from normal cells		Lipids from leucocidin-treated cells	
Procedure	Outline of procedure	P recovered (μg.)	Specific activity (counts/min./100 μg. of P)	P recovered (μg.)	Specific activity (counts/min./100 μg. of P)
A	10^9 cells washed in Hanks medium. Lipids extracted in chloroform–methanol and washed five times with upper-phase solvents	250	21	213	105
B	6×10^9 cells washed in Hanks medium. Lipids extracted in chloroform–methanol, passed over a cellulose column, dissolved in ether and washed with acid	1050	28	1140	190
C	9×10^8 cells washed five times with trichloroacetic acid. Lipids extracted in chloroform–methanol, dissolved in ether and washed with acid	225	27	245	170

times the radioactivity of the lipids of normal cells. When carrier-free orthophosphate was added to a sample of unlabelled lipids from normal leucocytes and put through the three purification procedures used here less than 0·003% of the added radioactivity was found in the product. The finding that the three different extraction and washing procedures give a similar result is taken as evidence that the radioactivity of the washed phospholipids is not due to contamination with non-lipid constituents.

Table 5 shows the distribution of radioactivity in fractions of the lipids separated on columns of silicic acid. The increased radioactivity of the lipids from the leucocidin-treated cells is associated with bands A and B. Karnovsky (1961) has found that during phagocytosis in guinea-pig leucocytes there is an increased rate of incorporation of ^{32}P into phosphatidic acid, phosphatidylserine and phosphatidylinositol. Comparison with the chromatographic separations achieved by Hanahan, Dittmer & Warashina (1957), and by Gray & Macfarlane (1958), with those achieved here makes it probable that phosphatidic acid would be present in band A and the nitrogenous lipids in bands C and D. There is no increased radioactivity associated with bands C, D and E; it is probable that these represent inositide, lecithin and sphingomyelin respectively.

Partition of phospholipids between chloroform-rich and methanol-rich phases. Table 4 indicated that the lipids obtained by procedure *A* (washing by the method of Folch *et al.* 1957) had a lower specific activity than those obtained by the other washing procedures. Consideration of the findings of Folch *et al.* (1957) made it probable that this was due to extraction of acidic lipids into the upper phase. The material extracted into the upper phase could be recovered, after acidification, by extraction with ether, which is taken as evidence for its lipid character. Table 6 shows that the upper-phase lipid has a high specific radioactivity and that the upper-phase lipid from leucocidin-treated cells is 17 times as radioactive as that from normal cells. On chromatography of the upper-phase lipid on silicic acid-impregnated paper 80% of the radioactivity was found associated with a material having R_F 0·7–0·8, and on elution the specific activities of the material from leucocidin-treated and normal leucocytes were 2500 and 110 counts/min./100 μg. of phosphorus respectively. The R_F of the highly radioactive material is consistent with

Table 5. *Effect of leucocidin on the incorporation of* ^{32}P *into some phospholipid fractions of the leucocyte*

The lipids derived from 10^{10} normal and 10^{10} leucocidin-treated leucocytes were fractionated on 10 g. of silicic acid.

		Leucocidin-treated cells		Normal cells	
Band	Possible constituents	Specific activity (counts/min./100 μg. of P)	Percentage of recovered P found in fraction	Specific activity (counts/min./100 μg. of P)	Percentage of recovered P found in fraction
A	Phosphatidic acid Polyglycerophosphatide	1200	5	100	6·5
B	Phosphatidylserine Phosphatidylethanolamine Kephalin plasmalogen	160	29·5	20	32
C	Inositide	23	11·5	19	9·5
D	Lecithin	16	33	15	35
E	Sphingomyelin	16	21	9	17

Table 6. *Distribution of phospholipid between two phases produced by partitioning*

An amount of lipid containing 1300 μg. of P from normal cells, and a similar amount from leucocidin-treated cells, after washing was dissolved in 20 ml. of chloroform–methanol (2:1, v/v), partitioned with 0·2% Na_2HPO_4 and the lower phase extracted five times with 10 vol. of chloroform–methanol–0·2% Na_2HPO_4 (3:48:47, by vol.).

	Normal cells		Leucocidin-treated cells	
Fraction	Specific activity (counts/min./100 μg. of P)	Percentage of recovered P found in fraction	Specific activity (counts/min./100 μg. of P)	Percentage of recovered P found in fraction
Lipids in upper phase	45	15	750	12·5
Lipids in lower phase	23	85	70	87·5

Table 7. *Incorporation of ^{32}P into the ribonucleic acids of leucocytes*

Duplicate suspensions containing 4×10^9 leucocytes were incubated with ^{32}P for 20 min.; leucocidin was added to one suspension and incubation continued for a further 20 min. The ribonucleic acids were extracted, hydrolysed and the nucleotides separated by ionophoresis on paper.

	Normal cells		Leucocidin-treated cells	
Nucleotide	P recovered (μg.)	Specific activity (counts/min./ 100 μg. of P)	P recovered (μg.)	Specific activity (counts/min./ 100 μg. of P)
Cytidylic acid	41	20	44	27
Adenylic acid	22	22	24	30
Guanylic acid	40	23	50	20
Uridylic acid	45	24	48	19

its having a phosphatidic acid character but quite inconsistent with its being a phosphoinositide (Hokin & Hokin, 1958). On chromatography of the upper-phase lipid on Dowex 1, 80% of the radioactivity was retained by the resin and 60% was recovered in the eluate in ethanolic hydrochloric acid. This is taken as further evidence for its acidic character.

Incorporation of ^{32}P into the ribonucleic acids of the leucocyte. In one case the effect of leucocidin on the incorporation of ^{32}P into the ribonucleic acids of the leucocyte was assessed. The results, shown in Table 7, indicate no significant difference between the rates of incorporation in normal and leucocidin-treated cells.

DISCUSSION

Woodin (1961) showed that the release of protein from the leucocyte during leucocidin action was not accompanied by a decrease in the amount of protein found in solution after homogenization of the cells. The present paper shows that homogenization by the procedure of Cohn & Hirsch (1960*a*) gives a similar result and also shows that corresponding to the release of protein is a decrease in the protein content of the granule fraction. It has not been possible to free the nuclear fraction of the homogenate of normal cells completely from granules and it is probable that the different protein contents of the nuclear fractions of normal and leucocidin-treated leucocytes reflect the higher granule content of the former.

Many of the components of the granules have been identified by Cohn & Hirsch (1960*a*), and, of these, lysozyme and phagocytin were previously found in the protein released by leucocidin (Woodin, 1961). To these can now be added ribonuclease, deoxyribonuclease, β-glucuronidase and peroxidase. The specific activities of the enzymes in the leucocidin-treated cell supernatant and in the granular fraction of normal cells are consistent with the bulk of the granule contents being transferred to the exterior of the cell. Only small amounts of the phosphatases are released from the cell by leucocidin and there is no direct evidence in explanation of this.

Although it cannot be proved that the protein released from the cell by leucocidin is derived exclusively from the granules the experimental evidence is consistent with this explanation and leaves no doubt that the bulk of the protein has this origin.

Observation with the phase-contrast microscope reveals that before they disappear the granules in the leucocidin-treated cell are in violent brownian movement (Gladstone & van Heyningen, 1957). This indicates an absence of morphological continuity of the granules with the cell surface. The possibility that the interior of the granules is continuous with the exterior of the cell through invaginations of the cell surface need not be considered. The release of protein from the leucocyte thus requires the passage of the granule contents through the cell surface membrane.

There is a parallel between the extrusion of protein from the leucocidin-treated leucocyte and the secretion of protein by the pancreas. The experiments of Heidenhain (1875) made it probable that the proteins secreted by the pancreas are derived from the zymogen granules of the acinus cells. Hokin (1955) isolated these granules and showed that they contain the enzymes present in the pancreatic secretion. Mainly on the basis of observations with the electron microscope, Palade (1959) has proposed that the extrusion phase of protein secretion involves the fusion of the granules with the cell membrane and liberation of the contents to the exterior.

It has been shown by Hokin & Hokin (1958) that the secretion of protein induced in the pancreas by acetylcholine is associated with an increased incorporation of ^{32}P into phospholipids, mainly localized in the phosphoinositide fraction. This effect was observed in pancreas slices incubated for over 2 hr. It has been shown in the present paper that the extrusion of protein from the leucocyte

during incubation with leucocidin for 10 min. is associated with a 20-fold increase in incorporation of ^{32}P into an acidic phospholipid distinct from the inositides. As the radioactivity of the phosphate-containing precursors of the leucocyte lipids has not been measured it is not certain that the increased incorporation represents an increased turnover of the phospholipids. However, as the increased metabolic activity leading to increased labelling of the phospholipids is induced during leucocidin action, and as reconstitution of the granules of the leucocyte does not occur, it is probable that the increased metabolic activity is associated with the actual extrusion of the proteins through the cell membrane.

There have been speculations that leucocytes may have a secretory function (cf. Richter, 1955), but direct demonstration of selective transfer of the proteins of the granules to the exterior of the cell does not appear to have been shown before. It is not at present clear if the release of protein occurs under other conditions or if the two components of leucocidin provide the cell surface with an enzymic equipment it otherwise lacks, permitting the transfer of protein to the exterior of the cell.

SUMMARY

1. The effect of leucocidin on the distribution of protein and nucleic acid in subcellular fractions of the leucocyte has been assessed.

2. Ribonuclease, deoxyribonuclease, β-glucuronidase and peroxidase have been found to be present in the proteins liberated from the leucocyte by leucocidin.

3. It is concluded that the protein released by leucocidin from the leucocyte is derived mainly if not entirely from the cell granules.

4. There is an increased incorporation of ^{32}P into the phospholipids of the leucocyte, which is largely localized in an acidic lipid distinct from phosphoinositide.

5. There is no increased incorporation of ^{32}P into the ribonucleic acids of the leucocyte during leucocidin action.

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Further Fractionations of Histones from Calf Thymus

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Calf-thymus histones have been separated into fractions relatively rich in lysine or arginine by many methods (Stedman & Stedman, 1951; Davison & Butler, 1954; Grégoire & Limozin, 1954; Crampton, Moore & Stein, 1955; Daly & Mirsky, 1955; Davison & Shooter, 1956; Luck, Rasmussen, Satake & Tsvetikov, 1958). Recently, Johns, Phillips, Simson & Butler (1960) have shown that by combining two previous chromatographic methods (Davison, 1957; Phillips & Johns, 1959)