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The Incorporation of Radioactive Phosphorus in the Leucocyte during the Extrusion of Protein Induced by Staphylococcal Leucocidin

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It has been shown that treatment of the polymorphonuclear leucocyte with leucocidin stimulates the incorporation of radioactive phosphorus into the phospholipids of the cell (Woodin, 1962). The radioactivity of the precursors was not known and so it was uncertain if this represented an increased turnover. The present paper shows that the stimulated incorporation of radioactive phosphorus is found in the nucleotides and other acid-soluble compounds of the leucocidin-treated leucocyte. There is no evidence for a causal connexion between this stimulated incorporation and the extrusion of protein from the granules of the leucocyte.

METHODS

The two components of leucocidin were crystallized as described by Woodin (1960); unless otherwise indicated they were added to cell suspensions to give $2\text{ }\mu\text{g./}10^8$ cells. Polymorphonuclear leucocytes were obtained from peritoneal exudates of rabbits (Woodin, 1962) and will be referred to below as leucocytes. ATP and ADP were obtained from C. F. Boehringer und Soehne, Mannheim, Germany; GTP and GDP were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. The solutions used for suspending the cells were Hanks (1948) medium or Krebs-Ringer solutions (Umbreit, Burris & Stauffer, 1957). Carrier-free [^{32}P]orthophosphate, [^{14}C]adenine sulphate and sodium [^{14}C]acetate were obtained from The Radiochemical Centre, Amersham, Bucks. The antileucocidin serum (staphylococcal antitoxin, CPP 76/63) was kindly provided by Miss Mollie Barr, Wellcome Research Laboratories, Beckenham, Kent.

Units. The definition of the L^+ unit and the unit of the antiserum CPP 76/63 is given by Woodin (1960).

Incorporation of ^{32}P into the nucleotides of the leucocyte. Tubes containing 5×10^8 cells in 10 ml. of Hanks solution were incubated for 10 min. at 37° and then leucocidin was added to one tube and ^{32}P (0.05 ml. containing 10^7 counts/min.) was added to both tubes. Incubation was continued for 10 min. and the tubes were cooled in ice. They were centrifuged at 0° for 5 min. at 2000 rev./min. and the supernatant was rejected. The cell pellets were extracted for 2 hr. at room temperature with 20 vol. of chloroform-methanol (2:1, v/v). The suspensions were centrifuged and the supernatants used for the preparation of the phospholipids (see below). The cell residues were washed with ether and dried in a stream of air. The powder was then extracted with 10 ml. of ice-cold HClO_4 (3%, w/v), residual ether and occluded air being removed by evacuating the tubes until the cell residues no longer floated on the surface. Extraction was continued for 30 min. and then the suspensions were centrifuged at 20 000 rev./min. for 20 min. The slightly opalescent supernatant was passed down a column containing 100 mg. of Norit and 100 mg. of Celite (Threlfall, 1957), which was then washed three times with 3 ml. of water. The effluent from the column was collected; it contained the nucleotide-free, acid-soluble fraction. The nucleotides were eluted from the washed Norit-Celite column in 7 ml. of pyridine and freeze-dried. The solid was dissolved in a small volume of water and put on paper for chromatography. After separation as described below the specific activities of the nucleotides were determined.

In a similar experiment, 2×10^8 leucocytes in 12 ml. of Hanks medium were incubated alone or with added leucocidin for 10 min. ^{32}P (0.1 ml.; 2×10^8 counts/min.) was added and incubation at 37° continued for 20 sec. The suspensions of cells were then diluted with 75 ml. of ice-cold Hanks medium and centrifuged at 0° . The nucleotides were isolated as described above and the specific radioactivities determined.

To determine the effect of antitoxin on the stimulated incorporation of ^{32}P into the nucleotides of the leucocidin-treated cell, two tubes each containing 2×10^8 cells in 7 ml.

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of Hanks medium were incubated at 37° for 10 min. To one tube 0.05 ml. of F component of leucocidin, containing 1.8 L^+ units (Woodin, 1960) and 0.05 ml. of S component of leucocidin, containing 17 L^+ units, were added and incubation of both tubes was continued for a further 10 min. To the tube containing the leucocidin-treated cells 0.3 ml. of antitoxin, containing 27.8 units of antileucocidin, was added and then to both tubes ^{32}P (2×10^6 counts/min.) was added and incubation continued for a further 10 min. The cell pellets were separated and the specific activities of the nucleotides determined.

Loss of radioactivity from nucleotides of labelled leucocytes incubated in a non-radioactive medium. Leucocytes (6.5×10^9 in 23 ml. of phosphate-free Krebs–Ringer bicarbonate solution) were incubated with ^{32}P (0.1 ml.; 9×10^7 counts/min.) for 15 min. at 37° . They were then diluted with 200 ml. of ice-cold Krebs–Ringer phosphate solution and centrifuged at 0° at 2000 rev./min. for 5 min. The cell pellet was washed twice in 200 ml. of ice-cold Krebs–Ringer phosphate solution and then suspended in Krebs–Ringer phosphate solution. The cell suspension was divided between three tubes, each of which contained 10 ml. of cell suspension. One tube was centrifuged at 0° immediately and the cell pellet isolated. The other tubes were incubated at 37° for 10 min., one with added leucocidin, and then treated in the same way. The nucleotides were isolated as described above and the specific radioactivities determined. They are not corrected for the radioactivity of the medium.

Incorporation of ^{32}P into phospholipids. The chloroform-methanol extract of the centrifuged cell pellets (see above) were washed by 'method B' of Woodin (1962). The phosphorus and total radioactivity of the product were determined.

Effect of glycolysis inhibitors and calcium on incorporation of ^{32}P into the nucleotides and phospholipids of the leucocyte. To determine the effect of glycolysis inhibitors, tubes containing 3×10^9 leucocytes in 10 ml. of Hanks medium containing 25 mM-sodium fluoride or 0.01 M-sodium arsenate or 0.04 mM-sodium iodoacetate were incubated with leucocidin and ^{32}P (1.0 ml.; 5×10^6 counts/min.) for 10 min. at 37° . Control tubes containing no inhibitors were treated in the same way. After incubation the tubes were centrifuged at 0° , the phospholipids and nucleotides in the cell pellets were separated and the specific radioactivities determined.

The effect of calcium on the incorporation of ^{32}P was assessed by incubating 2×10^9 normal and leucocidin-treated leucocytes in 12.5 ml. of Hanks medium containing 3 mg. of Ca-EDTA or in 12.5 ml. of calcium-free Hanks medium containing 3 mg. of Mg-EDTA with ^{32}P (0.05 ml.; 6×10^6 counts/min.) for 10 min. at 37° . The tubes were centrifuged at 0° , the nucleotides and phospholipids in the cell pellets were separated and their specific radioactivities determined.

Incorporation of ^{14}C adenine into the nucleotides. Two tubes each containing 2×10^9 cells in Hanks solution were incubated at 37° for 10 min. with 0.19 mg. of ^{14}C adenine containing 297 000 counts/min. The nucleotides were isolated as described above.

Incorporation of ^{14}C acetate into the lipids. Tubes containing 2×10^9 cells in 6 ml. of Hanks medium were incubated for 10 min. at 37° with $34 \mu\text{g}$. of sodium [^{14}C]acetate containing 125 000 counts/min. The chloroform-methanol

extract was prepared as described above but in the washing with acid (method B of Woodin, 1962) 0.1 N-acetic acid was substituted for 0.1 N-HCl.

Hydrolysis of the esterified phosphate in the nucleotide-free acid-soluble extract of the leucocytes. The nucleotide-free acid-soluble fraction of the leucocytes is the material unadsorbed on the Norit–Celite columns used in the preparation of the nucleotide fraction (see above). Samples of the nucleotide-free acid-soluble fraction (10 ml.) from 2×10^9 normal and 2×10^9 leucocidin-treated cells, which had been incubated for 20 sec. with ^{32}P (1.7×10^7 counts/min.), were prepared. A portion (1 ml.) was treated by the ammonium molybdate–2-methylpropan-1-ol method to remove orthophosphate (see below) and the residual phosphorus content determined. A further 1 ml. volume was treated by the ammonium molybdate–2-methylpropan-1-ol method with the addition of extra orthophosphate (see below) and the residual radioactivity determined. Further 1 ml. samples were hydrolysed: (a) in $\text{N-H}_2\text{SO}_4$ for 10 min. at 100° ; (b) in $\text{N-H}_2\text{SO}_4$ for 2 hr. at 100° ; (c) in N-NaOH for 20 min. at 20° ; (d) in 0.2 N-NaOH containing iodine (see Umbreit *et al.* 1957; p. 278). After hydrolysis, the samples were treated with ammonium molybdate and 2-methylpropan-1-ol and the residual radioactivity and phosphorus determined.

Radioactivity of the inorganic phosphorus of the cell. Six tubes each containing 8×10^8 cells in 5 ml. of Hanks solution were prepared. To three tubes leucocidin was added. To one of the leucocidin-treated cell suspensions and to one with a suspension of normal cells, ^{32}P (0.1 ml.; 150 000 counts/min.) was added, and the suspensions were incubated for 10 min. at 37° and cooled in ice. The other four tubes were incubated for 10 min. at 37° and then one suspension of leucocidin-treated cells and one suspension of normal cells were cooled in ice; ^{32}P (0.1 ml.; 150 000 counts/min.) was added to all four tubes and the temperature conditions were maintained (two tubes at 0° and two tubes at 37°) for 10 min. Then all the cell suspensions were diluted with 250 ml. of ice-cold Hanks medium and centrifuged at 2000 rev./min. for 7 min. The cell pellets were washed in the same way with a further 250 ml. of Hanks medium and then with 250 ml. of 0.9% NaCl solution. The washed cell pellets were then extracted with 5 ml. of 10% (w/v) trichloroacetic acid for 30 min. and the insoluble material was centrifuged off. The orthophosphate in the soluble fraction was precipitated by the method of Delory (1938) and determined by the method of Berenblum & Chain (1938). A sample of the 2-methylpropan-1-ol extract obtained in the latter method was used to determine the radioactivity.

Rate of diffusion of orthophosphate from the leucocyte. Two suspensions of 2×10^9 cells in Hanks solution were incubated at 37° for 10 min., one containing added leucocidin. They were then diluted with 250 ml. of ice-cold phosphate-free Krebs–Ringer bicarbonate solution and centrifuged at 0° . The cell pellets were suspended in 50 ml. of phosphate-free Krebs–Ringer bicarbonate solution and incubated at 0° . Duplicate samples (5 ml.) were withdrawn from each suspension immediately and after 5, 20 and 40 min. at 0° . One sample was placed in a weighed tube, centrifuged at 3000 rev./min. for 3 min. and the supernatant rejected. The wet and dry weights of the cell pellet were determined. The other sample was centrifuged in the same way and the supernatant precipitated with 0.5 ml. of

100% (w/v) trichloroacetic acid. The cell pellet was extracted with 5 ml. of 10% (w/v) trichloroacetic acid. The inorganic P content of the trichloroacetic acid extracts was determined. The concentration of inorganic P in the cell was calculated by assuming the extracellular volumes in the pellets of normal and leucocidin-treated cells to be 25 and 55% respectively (Woodin & Wieneke, 1963).

Effect of antitoxin on the synergism of the two components of leucocidin. Seven tubes were prepared with 1.2×10^8 cells in 2.5 ml. of Hanks solution and the amounts of the two components of leucocidin listed in Table 7 under the heading 'First additions'. They were incubated at 37° for 10 min. and centrifuged. The supernatant of tube 6 (which contained both components of leucocidin) was collected and the cell pellet rejected. The supernatants of the other tubes were rejected and the cell pellets suspended in 5 ml. of Hanks solution containing in some cases sufficient antitoxin (see Table 7: 'Second additions') to neutralize the leucocidin already added. The tubes were incubated at room temperature for 5 min. and centrifuged. The supernatants were rejected and the cell pellets suspended in 2.5 ml. of Hanks solution to which had been added the amounts of the two components of leucocidin indicated in Table 7 as 'Third additions'. The tubes were incubated at 37° for 10 min. and centrifuged. The β -glucuronidase in a sample (0.3 ml.) of the cell supernatant was determined.

Determination of radioactivity. The radioactivity of phospholipids containing ^{32}P was determined as described by Woodin (1962). For the determination of radioactivity of lipids or nucleotides containing ^{14}C , samples containing less than 200 μg . of solid were dried on planchets (surface area 3.9 cm^2) and taken as at infinite thinness. For the determination of the radioactivity of nucleotides containing ^{32}P , samples (0.5 ml.) of the eluates of the chromatograms (see below) were mixed with 0.1 ml. of 10N- H_2SO_4 , 0.2 ml. of 5% (w/v) ammonium molybdate, 0.2 ml. of 0.2% (w/v) NaH_2PO_4 and 3 ml. of 2-methylpropan-1-ol and shaken in stoppered tubes for a few minutes. The alcohol layer was rejected and a further 0.2 ml. of 0.2% (w/v) NaH_2PO_4 added to the aqueous phase, which was shaken with a further 3 ml. of 2-methylpropan-1-ol. This was repeated. The aqueous phase was then made alkaline to phenolphthalein and dried on a planchet. This procedure ensured freedom from radioactive orthophosphate. The same procedure was used with samples of the nucleotide-free acid-soluble fraction. To determine the radioactivity of the orthophosphate of the leucocyte the final 2-methylpropan-1-ol layer obtained in the Berenblum & Chain (1938) method for the quantitative determination of the orthophosphate was made alkaline and 0.5 ml. dried on a planchet. The specific radioactivities reported in this paper are corrected to an activity of 10^5 counts/min./ml. of medium.

Separation of nucleotides by paper chromatography. Descending chromatography on Whatman no. 1 paper, prepared by the method of Eggleston & Hems (1952), was used. The solvents were isobutyric acid-ammonium isobutyrate (Krebs & Hems, 1952) and propanol-aq. NH_3 solution (Heald, 1957). After development the papers were dried at room temperature and the position of the nucleotides determined by inspection under ultraviolet light (Hanovia, Chromatolite). The nucleotides were eluted in 3 ml. of water.

Analytical methods. The determination and the unit of

β -glucuronidase is described by Woodin & Wieneke (1963). Inorganic, total and acid-labile phosphorus were determined by the Berenblum & Chain (1938) method after hydrolysis or digestion as described by Woodin (1961). Esterified fatty acid was determined by the method of Morgan & Kingsbury (1959). Chromatography of nucleotides on Dowex 1 (X8) was by the method of Hurlbert, Schmitz, Brumm & Potter (1954); the ammonium formate-formic acid elution system was used. For the determination of the distribution of radioactivity in ATP, 0.6 μmole of ATP (containing 2244 counts/min.) obtained from normal leucocytes and 0.4 μmole of ATP (containing 14492 counts/min.) obtained from leucocidin-treated cells were each mixed with 1.5 μmoles of unlabelled ATP and incubated with hexokinase (C. F. Boehringer und Soehne) under the conditions described by Crane & Sols (1955). The reaction was stopped by adding 0.1 vol. of 30% (w/v) HClO_4 and the nucleotides were adsorbed on Norit-Celite (see above). The Norit-Celite was eluted as described above and the nucleotides were separated by paper chromatography. The specific activities of the ADP and the glucose 6-phosphate in the Norit-Celite effluent were determined.

RESULTS

Incorporation of ^{32}P into nucleotides of the leucocyte. Chromatography of the nucleotide fraction in isobutyric acid-ammonium isobutyrate gave a good separation into spots that contained ATP, ADP, AMP, GTP and GDP respectively, but chromatography of the eluates of the spots containing the adenine nucleotides in propan-2-ol-ammonia showed that these spots contained additional material, which adsorbed strongly in the ultraviolet. However, none of this additional material contained acid-labile phosphorus and none was highly radioactive when obtained from cells incubated in the presence of ^{32}P . Accordingly, the specific activities of the individual nucleotides were determined from the radioactivity and acid-labile phosphorus content of spots obtained after one-dimensional chromatography in isobutyric acid-ammonium isobutyrate.

The identification of the guanine nucleotides was based upon their spectra, their acid-labile phosphorus content and upon their migrating on paper chromatograms to positions similar to those of the authentic nucleotides. The identity of the radioactive material in the ATP and the ADP spots was confirmed by addition of authentic nucleotide and chromatography on Dowex 1; 95–100% of the radioactivity was recovered in the elution volume containing the authentic nucleotide.

Table 1 gives the specific radioactivities of the nucleotides obtained from normal and leucocidin-treated cells. Duplicate determinations of the specific activity differed by less than 15% but there was considerable variation in the amount of the individual nucleotides recovered. The amounts of the nucleotides recovered, reported in Table 1,

Table 1. *Incorporation of ^{32}P into some nucleotides of normal and leucocidin-treated leucocytes*

Material	ATP		ADP		GTP		GDP	
	Amount recovered ($\mu\text{mole}/10^8$ cells)	Sp. activity (counts/min./ μmole)	Amount recovered ($\mu\text{mole}/10^8$ cells)	Sp. activity (counts/min./ μmole)	Amount recovered ($\mu\text{mole}/10^8$ cells)	Sp. activity (counts/min./ μmole)	Amount recovered ($\mu\text{mole}/10^8$ cells)	Sp. activity (counts/min./ μmole)
Normal cells, 10 min. incubation with ^{32}P	0.012	730	0.004	640	0.0026	530	0.0015	420
Leucocidin-treated cells, 10 min. incubation with ^{32}P	0.0025	34 000	0.009	17 000	0.00076	11 500	0.00085	8500
Normal cells, 20 sec. incubation with ^{32}P	0.016	1.08	0.008	0.8	—	—	—	—
Leucocidin-treated cells, 20 sec. incubation with ^{32}P	0.0052	25	0.019	12	—	—	—	—

Normal and leucocidin-treated leucocytes were incubated with ^{32}P for the time indicated and the nucleotides extracted from the centrifuged cell pellets and separated by paper chromatography.

indicate the amount of material available for determination of the specific activity and not the amounts in the cells. The specific activity of the AMP of the leucocidin-treated cell was less than twice that of the normal cell. There was no stimulated incorporation of ^{32}P into the nucleotides when the leucocytes were treated with either component of leucocidin alone.

Leucocidin induces swelling and uptake of water by the leucocyte, which can be inhibited by adding dextran (5 %, w/v) to the medium (Woodin, 1961). This concentration of dextran had no effect on the incorporation of ^{32}P into the nucleotides of the leucocidin-treated cell.

Treatment with hexokinase indicated that the β - and γ -phosphorus atoms of ATP (derived from either normal or leucocidin-treated cells) were equally labelled and together contained 90 % of the total radioactivity. Treatment with hydrochloric acid indicated that the α -phosphorus atom of ADP contained less than 10 % of the total radioactivity of the ADP from either normal or leucocidin-treated cells. The same distribution of radioactivity between the phosphorus atoms of ATP and ADP was obtained for material from cells that had been labelled for 10 min. or 20 sec.

Table 2 shows that if cells previously labelled with ^{32}P are incubated in a non-radioactive medium there is a reduced rate of loss of radioactivity from the nucleotides in the leucocidin-treated cell.

Incorporation of ^{32}P into the nucleotide-free acid-soluble fraction of the leucocyte. Leucocidin stimulated incorporation of ^{32}P into the other acid-soluble compounds of the leucocyte. After 20 sec. labelling the nucleotide-free, acid-soluble fraction of normal and leucocidin-treated cells had 0.3 and 4.8 counts/min./ μmole of phosphorus respectively. The individual compounds in this fraction were not separated but differential hydrolysis indicated a similar proportion of the radioactivity to be hydrolysed from the fractions derived from normal and leucocidin-treated cells (Table 3).

Effect of metabolic inhibitors on the incorporation of ^{32}P in the leucocyte. It was possible that the incorporation of ^{32}P into the nucleotides and phospholipids of the leucocidin-treated cells occurred by different pathways and that an increased phospholipid-orthophosphate exchange took place as well as increased incorporation of ^{32}P into the nucleotides. Table 4 shows that three metabolic inhibitors known to affect the synthesis of ATP by glycolysis have a greater inhibitory effect on the incorporation into phospholipids than into nucleotides. These metabolic inhibitors do not affect the amount of β -glucuronidase liberated from the leucocidin-treated leucocyte.

It is shown in the next paper (Woodin & Wieneke, 1963) that omission of calcium from the medium

Table 2. *Effect of leucocidin on the loss of radioactivity from the nucleotides of leucocytes labelled with ^{32}P*

Leucocytes (6.5×10^9) were labelled by incubation with ^{32}P (9×10^7 counts/min.) in phosphate-free Krebs-Ringer bicarbonate solution. They were washed to remove external radioactivity and divided into three samples: (A) was immediately cooled in ice; (B) was incubated at 37° for 10 min. and cooled in ice; (C) was incubated with leucocidin at 37° for 10 min. and cooled in ice. Nucleotides were extracted from the centrifuged cell pellets. Specific activities are not corrected for the radioactivity of the medium.

Material	ATP		ADP	
	Amount recovered (μmole)	$10^{-3} \times \text{Sp. activity}$ (counts/min./ μmole)	Amount recovered (μmole)	$10^{-3} \times \text{Sp. activity}$ (counts/min./ μmole)
(A) Normal cells not incubated at 37°	0.051	100	0.035	38
(B) Normal cells incubated at 37°	0.086	62	0.041	15
(C) Leucocidin-treated cells incubated at 37°	0.013	82	0.021	30

Table 3. *Effect of hydrolysis on the radioactivity of the organic phosphorus in the nucleotide-free, acid-soluble fraction of the leucocyte*

The acid-soluble, nucleotide-free fraction from normal and leucocidin-treated leucocytes that had been incubated with ^{32}P for 20 sec. was treated as indicated and the inorganic phosphorus removed by treatment with ammonium molybdate and 2-methylpropan-1-ol. The residual radioactivity and phosphorus were determined. The normal cell extract (10 ml.) contained 97 $\mu\text{g.}$ of esterified phosphorus and the leucocidin-treated cell extract (10 ml.) contained 45 $\mu\text{g.}$ of esterified phosphorus.

Hydrolysis conditions	Radioactivity recovered (%)		Combined phosphorus recovered (%)	
	Normal cells	Leucocidin-treated cells	Normal cells	Leucocidin-treated cells
$\text{N-H}_2\text{SO}_4$, 100°	54	58	89	85
$\text{N-H}_2\text{SO}_4$, 100°	26	30	68	73
N-NaOH , 20 min. at 20°	95	98	—	—
I_2 in 0.2N-NaOH , 20 min. at 20°	89	100	—	—

Table 4. *Effect of glycolysis inhibitors on the incorporation of ^{32}P into the nucleotides and phospholipids of the leucocidin-treated leucocyte*

Cells were incubated with leucocidin for 10 min. at 37° in Hanks medium containing ^{32}P , to which the inhibitors had been added. The phospholipids and nucleotides in the cell pellets were isolated and the specific radioactivities determined. Results are presented as the ratio sp. activity without inhibitor/sp. activity with inhibitor.

Inhibitor	Ratio		
	ATP	ADP	Phospholipids
Fluoride (25 mM)	2.0	2.8	2.75
Iodoacetate (0.4 mM)	1.9	1.6	3.9
Arsenate (0.01 M)	3.3	3.0	4.4

inhibits the extrusion of protein from the leucocidin-treated leucocyte. Table 5 shows that the omission of calcium stimulates the incorporation of ^{32}P into the phospholipids. There is a decreased incorporation into the nucleotides of the leucocidin-treated cell in the absence of calcium.

Effect of leucocidin on the exchange and rate of release of the orthophosphate of the leucocyte. The accumulation of inorganic phosphate in the leucocidin-treated leucocyte has been described

(Woodin, 1961). Fig. 1 shows that very little difference in rate of diffusion of orthophosphate from the normal and leucocidin-treated cell can be detected. Table 6 shows that there is little difference in the specific radioactivity of the orthophosphate of the normal and leucocidin-treated cell and that the same proportion of the uptake is inhibited by low temperature. The increased incorporation of ^{32}P in the leucocidin-treated leucocyte is thus not due to increased permeability of the leucocidin-treated cell to orthophosphate.

Incorporation of ^{14}C adenine or ^{14}C acetate in the leucocyte. When incubated in the presence of ^{14}C adenine the specific activities of the ATP of the normal and leucocidin-treated cell were 12×10^3 and 9.7×10^3 counts/min./ μmole respectively and the specific activities of the ADP of the normal and leucocidin-treated cell were 8.6×10^3 and 9.0×10^3 counts/min./ μmole respectively.

After incubation with ^{14}C acetate the specific activities of the lipids of the normal and leucocidin-treated cell were 112 and 27 counts/min./ $\mu\text{equiv.}$ of esterified fatty acid respectively.

Effect of antitoxin on the synergism of the two components of leucocidin and on the stimulated incorporation of ^{32}P induced in the leucocyte by leuco-

Table 5. *Influence of calcium on the incorporation of ^{32}P into the nucleotides and phospholipids of the leucocyte*

Cells were washed and suspended in Krebs-Ringer bicarbonate solution containing 24 mg. of Ca-EDTA/100 ml. or in calcium-free Krebs-Ringer bicarbonate solution containing 24 mg. of Mg-EDTA/100 ml. After incubation with ^{32}P for 10 min. the nucleotides and phospholipids were isolated.

		ATP		ADP		Phospholipids	
Material		Amount recovered ($\mu\text{mole}/10^8$ cells)	Sp. activity (counts/min./ μmole)	Amount recovered ($\mu\text{mole}/10^8$ cells)	Sp. activity (counts/min./ μmole)	Amount recovered (μmole of P/ 10^8 cells)	Sp. activity (counts/min./ μmole of P)
Normal cells	With calcium	0.0075	730	0.004	640	0.85	4.0
	Without calcium	0.0055	1 700	0.0024	1 600	0.82	21
Leucocidin-treated cells	With calcium	0.0042	34 000	0.13	16 000	0.91	32
	Without calcium	0.0009	8 700	0.0017	3 000	0.81	100

Table 6. *Effect of incubation with leucocidin and at low temperature on the radioactivity of the inorganic phosphorus of the leucocyte*

After incubation with ^{32}P in Hanks medium as indicated, the cells were washed twice in Hanks medium and once in 0.9% NaCl solution. The cell pellets were extracted with trichloroacetic acid and the inorganic phosphate was isolated and its radioactivity determined.

Incubation conditions	Normal cells		Leucocidin-treated cells	
	Orthophosphate recovered ($\mu\text{mole}/10^8$ cells)	Sp. activity (counts/min./ μmole)	Orthophosphate recovered ($\mu\text{mole}/10^8$ cells)	Sp. activity (counts/min./ μmole)
10 min. at 37° with ^{32}P	0.105	1300	0.225	1100
10 min. at 37° and then 10 min. at 37° with ^{32}P	0.085	640	0.195	600
10 min. at 37° and then 10 min. at 0° with ^{32}P	0.095	290	0.235	260

Fig. 1. Permeability of leucocytes to orthophosphate. O, Normal leucocytes; Δ , leucocidin-treated leucocytes. Results are plotted as the ratio of concentrations: inorganic P in supernatant/inorganic P in cells. For details see text.

cidin. It was possible that the stimulated incorporation of ^{32}P arose from a phosphorylating reaction on the cell surface catalysed by the two components of leucocidin. The adsorption of the two components of leucocidin by the leucocyte has been described (Woodin, 1961). Table 7 indicates that after adsorption by the cell each component can be neutralized by antibody. When an excess of

antibody was added to the leucocidin-treated leucocytes before the addition of ^{32}P there was no reduction in the incorporation of radioactivity into the nucleotides.

DISCUSSION

The stimulated incorporation of ^{32}P in the leucocidin-treated leucocyte is not restricted to the phospholipids but is a general phenomenon affecting the nucleotides and other acid-soluble compounds of the cell. The most highly radioactive lipid fraction of the leucocyte had a chromatographic behaviour consistent with its being a phosphatidic acid or polyglycerolphosphatide. When leucocytes were treated with leucocidin this fraction showed a 22-fold-increased incorporation of ^{32}P (Woodin, 1962). The corresponding increase for the adenosine triphosphate of the cell is 46-fold. It is probable that the acidic lipid fraction has a decreased rate of turnover or exchange when the leucocyte is treated with leucocidin. The leucocidin-treated cell exhibits a decreased rate of several other metabolic reactions. The inhibition of glycolysis and oxygen uptake has been reported (Woodin, 1961) and in the present paper it has been shown that the incorporation of [^{14}C]adenine into ATP is smaller and the incorporation of [^{14}C]acetate into lipids is

Table 7. *Inhibition of adsorbed leucocidin by antibody*

Cells (1.2×10^6) were incubated in Hanks solution containing the 'first additions', centrifuged and suspended in Hanks solution containing the 'second additions', centrifuged and suspended in Hanks solution containing the 'third additions'. After incubation for 10 min. the supernatant was analysed for β -glucuronidase. The cells in tube 6 were put through the first incubation only.

	Tube no.						
	1	2	3	4	5	6	7
First additions	0	F component (3.4 L^+)	F component (3.4 L^+)	S component (20 L^+)	S component (20 L^+)	S component (20 L^+) and F component (3.4 L^+)	0
Second additions	Antibody (20 units)	0	Antibody (20 units)	0	Antibody (20 units)	—	Antibody (20 units)
Third additions	0	S component (25 L^+)	S component (25 L^+)	0	F component (13.5 L^+)	—	S component (25 L^+) and F component (13.5 L^+)
Units of β -glucuronidase in final supernatant	5	100	5	92	5	140	120

decreased fourfold when the leucocyte is treated with leucocidin. It has also been shown in the present paper that the leucocidin-treated cell shows a decrease in the rate at which radioactivity is lost from the nucleotides when cells labelled with ^{32}P are transferred to a medium of low specific activity. In view of these considerations we have no evidence that the increased incorporation of ^{32}P represents an increased rate of turnover of a phosphorus compound in the leucocidin-treated leucocyte.

It has been shown that the leucocidin-treated cell is no more permeable than the normal cell to orthophosphate and that the internal orthophosphate has a similar radioactivity to that of the normal cell. However, the possibility that in the leucocidin-treated cell the external orthophosphate is utilized directly by an exchange reaction at the cell surface is not excluded. Because of the failure of antileucocidin to inhibit the incorporation of ^{32}P in the leucocidin-treated cell the participation of the two components of leucocidin is not involved. The appearance of a new exchange reaction need not be hypothesized, for the process of fusion of the surface of the vesicles with that of the cell surface (Woodin, French & Marchesi, 1963) will result in a spatial rearrangement of molecules in these surfaces and it is possible that by this means direct utilization of external orthophosphate is facilitated.

SUMMARY

1. Incubation with leucocidin stimulates the incorporation of ^{32}P into the adenosine tri- and di-phosphate and guanosine tri- and di-phosphate of the leucocyte.

2. Incubation with leucocidin does not increase the permeability of the leucocyte to orthophosphate and in the presence of ^{32}P does not increase the radioactivity of the intracellular orthophosphate.

3. In the presence of fluoride, iodoacetate or arsenate the incorporation of ^{32}P into the lipids and nucleotides of the leucocidin-treated leucocyte is partially inhibited. The extrusion of β -glucuronidase is not affected under these conditions.

4. Omission of calcium from the medium stimulates the incorporation of ^{32}P into the lipids of the leucocidin-treated leucocyte and reduces the amount of incorporation into the nucleotides.

5. Either component of leucocidin adsorbed on the leucocyte can be neutralized by antibody. Addition of antibody to the leucocidin-treated leucocyte does not inhibit the incorporation of ^{32}P into the nucleotides.

6. Treatment with leucocidin decreases the rate of loss of radioactivity from the nucleotides of leucocytes labelled with ^{32}P and suspended in a medium of low specific radioactivity.

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 1.5×10^8 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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