Composition and Properties of a Cell-Membrane Fraction from the Polymorphonuclear Leucocyte

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1. The composition of a vesicular cell-membrane fraction from leucocytes has been studied. The bulk of the mass is accounted for as protein and lipid. A small amount of carbohydrate, including some *N*-acetylnauraminic acid, is present. The phospholipid/cholesterol molar ratio is 1.4 and differs from that for the whole cell. 2. Labile phosphorus groups are present in the membrane but the analysis is complicated by the presence of phosphorus occluded in the membrane vesicles. 3. Leucocidin does not change the gross composition of the membranes or alter the amount or reactivity of the phosphorus compounds. 4. The cell-membrane fraction has considerable avidity for an impurity present in commercial [^{32}P]-orthophosphate. When this is removed [^{32}P]orthophosphate or [^{32}P]ATP does not label the membrane. 5. The presence of an NADH_2 -cytochrome *c* oxidoreductase and an alkaline phosphatase is described. The adenosine-triphosphatase activity of the membrane has not been found to depend on the presence of Na^+ or K^+ .

The study of the interaction of leucocidin with the polymorphonuclear leucocyte has enabled the purification of material derived from the cell surface membrane (Woodin & Wieneke, 1966). The purified product consists of vesicles and broken membranes only. The leucocyte has little endoplasmic reticulum and the granules remain intact when the cells are homogenized, so that it is probable that the membrane fraction prepared by Woodin & Wieneke (1966) consists of cell surface membrane, some nuclear membrane and material from the Golgi apparatus only.

The present paper contains data on the composition and on the enzymic activities of the cell-membrane fraction. Because of their high rates of turnover and participation in cell metabolism phosphorus compounds have been assigned functions in mechanisms of transport and permeability control. Leucocidin induces in the leucocyte changes in permeability and phosphorus metabolism. The present paper contains some preliminary investigations of the stability of phosphorus compounds in the leucocyte membrane.

METHODS

Materials. The preparation of leucocidin, leucocytes, mixed membranes and light membranes was by the methods used by Woodin & Wieneke (1966). Krebs-Ringer media were prepared as described by Umbreit, Burris & Stauffer (1957).

The source of the radioactive phosphorus preparations

was the [^{32}P]P_i in dilute HCl listed as 'PBS1' by The Radiochemical Centre, Amersham, Bucks. Samples containing 20 mc in 0.5 ml. were purchased and diluted to 5 ml. with water. This preparation of [^{32}P]P_i is referred to in this paper as 'PBS1'. Partial purification of the commercial product was based on the procedure of Suelter, DeLuca, Peter & Boyer (1961).

A sample of 'PBS1' (1.0 ml. containing 4 mc) was treated with 1.88 ml. of 0.01 *N*-NH₃ solution and 0.08 ml. of 2 mM-K₂HPO₄ solution and diluted to 3 ml. with water. The solution was passed down a column (2 cm. $\times$ 0.5 cm.) of Dowex 1 (X8; HCO₃⁻ form). The column was washed with 200 ml. of water. The effluent contained 0.15 mc of radioactivity and was rejected. [The radioactivity was not adsorbed if it was passed through a further column of Dowex 1 (X8).]

The original column was eluted with 50 ml. of an aqueous solution containing 375 mg. of (NH₄)₂CO₃ and 0.65 ml. of *N*-NH₃. The eluate contained 70% of the added radioactivity and was treated with Zeo-Karb 225. A suspension containing 20 ml. packed volume of Zeo-Karb 225 (H⁺ form) was sucked dry on a filter paper and the resin added to the effluent from the Dowex column. When evolution of CO₂ had stopped the resin was filtered off on a sintered-glass filter. The filtrate was concentrated to about 5 ml. by evaporation in a beaker under a heat lamp and then adjusted to pH 7-7.5 with 0.1 *N*-NaOH. The product contained 50-60% of the initial radioactivity. This material is referred to below as 'partially purified PBS1'. 'Highly purified PBS1' was prepared from this by drying the solution in a desiccator, dissolving the solid in 0.5 ml. of 0.01 *M*-tris buffer, pH 7.2, and fractionating the solution on a column containing 1 g. of Sephadex G-25 with 0.01 *M*-tris buffer, pH 7.2, as eluent. The radioactivity in 0.5 ml. fractions was

determined and the first 70% of the radioactivity rejected. The remainder is the 'highly purified PBS1'.

[^{32}P]ATP was prepared from 'PBS1' by the method of Glynn & Chappell (1964).

Buffers. Tris buffers, unless otherwise stated, were chloride salts and phosphate buffers were potassium salts.

Chemical methods. Nucleic acids were separated from subcellular fractions by extraction in 0.45 *N*-HClO₄ at 90° for 15 min. DNA in the extract was determined by the method of Burton (1956), and RNA in the extract was determined by the orcinol reaction described by Markham (1955). Protein was determined colorimetrically (Lowry, Rosebrough, Farr & Randall, 1951). Other methods were: cholesterol (Henly, 1957); esterified fatty acid (Morgan & Kingsbury, 1959); total carbohydrate by the anthrone reaction (Dische, 1955); *N*-acetylneuraminic acid (Warren, 1959); phosphorus (Berenblum & Chain, 1938). The determination and definition of membrane units is given by Woodin & Wieneke (1966).

To determine NADH₂-cytochrome *c* oxidoreductase 3 ml. of 0.1 *M*-tris buffer, pH 7.2, containing 1 mg. of cytochrome *c*, 0.75 μmole of NADH₂ and 0.1–0.5 membrane unit was maintained at room temperature and changes in $E_{550\text{ m}\mu}$ were determined. NADPH-cytochrome *c* oxidoreductase was determined similarly with 0.75 μmole of NADPH₂ and 1.0 unit of membranes. The unit of these enzymes is the amount reducing 1 μmole of cytochrome *c*/min. under the above conditions. To detect oxidation of NADH₂ or NADPH₂ 0.3 μmole of these was maintained at room temperature in 3.0 ml. of 0.03 *M*-tris buffer, pH 7.2, with 1 unit of membranes and changes in $E_{340\text{ m}\mu}$ were determined. In some cases mixtures also contained menadione sodium bisulphite (sodium 2-methyl-1,4-naphthoquinone-2-sulphonate) (0.3 *mm*).

Determination of radioactivity. Solid samples were counted at infinite thinness with a Geiger-Müller end-window tube. With this detector 1 mc contained 2×10^8 counts/min.

Centrifugations. Centrifugation at 1500 *g* was done in an MSE Major centrifuge (Measuring and Scientific Equipment Ltd., Crawley, Sussex). Centrifugation at 16000 *g* was done in the angle head of the MSE 17 centrifuge (Measuring and Scientific Equipment Ltd.). Centrifugation at 25000 *g* was done in the no. 40.2 angle head of the Spinco model L ultra-centrifuge.

Composition of light membranes. Light membranes were prepared from 9×10^9 cells and dialysed against water [adjusted to pH 7.2 with 4% (w/v) NaHCO₃ solution] to give 7.7 ml. of suspension. Samples (0.05 ml.) were taken for the determination of the $E_{280\text{ m}\mu}$ (in 0.2% sodium deoxycholate-0.1 *N*-NaOH solution) for the determination of protein (0.1 ml.) and other samples (0.5 ml.) for the determination of the weight after drying at 110° or the nitrogen content. To prepare the lipid extract 4 ml. of membrane suspension were freeze-dried in a weighed tube and the solid extracted with 5 ml. of chloroform-methanol (2:1, v/v). The extract was removed from the insoluble material and shaken with 0.2 vol. of 0.04% CaCl₂ solution, and the upper layer, which separated on standing, was rejected. The lower layer was analysed for cholesterol, phosphorus, total nitrogen and esterified fatty acid. The material insoluble in chloroform-methanol was washed with ether and dried over P₂O₅. The dry weight and nitrogen content were determined. A further sample of light membranes (2.0 ml.) was treated in the same way to give the chloroform-methanol- and ether-

insoluble material and this was dried and dissolved in 0.1 *N*-NaOH. Samples of this were used to determine the protein content and the phosphorus content.

The carbohydrate content of three samples of light membranes was determined. To remove free sucrose, light membranes were concentrated by centrifugation (Woodin & Wieneke, 1966) and 0.5 ml., containing 8–12 units, was passed down a column containing 4 g. of Sephadex G-25 in 0.01 *M*-tris buffer, pH 7.2. The $E_{280\text{ m}\mu}$ value and the anthrone-positive material were determined on the eluted fractions (0.5 ml.) and clear separation of the membrane peak from the sucrose peak was obtained. The fractions containing the membranes were combined; they contained 70% of the ultraviolet-absorbing material added to the columns. *N*-Acetylneuraminic acid and anthrone-positive material were determined in the membrane fraction. In a further case membranes were prepared in this way and 10 units incubated alone or with 50 μg . of each component of leucocidin at 37° for 10 min. The mixtures were centrifuged at 25000 *g* for 30 min. and the supernatant was analysed for *N*-acetylneuraminic acid and anthrone-positive material. To determine the association of carbohydrate with the lipid components of the membrane 8 units of light membranes were prepared free from sucrose and centrifuged at 16000 *g* for 30 min. in thick glass tubes. The pellet was extracted with 5 ml. of chloroform-methanol (2:1, v/v) and then with 5 ml. of ether. The *N*-acetylneuraminic acid and anthrone-positive material were determined on the soluble fraction and on the suspension obtained by adding 0.6 ml. of 0.1 *N*-H₂SO₄ to the insoluble residue.

Distribution of phosphorus in light membranes. Light membranes were dialysed overnight against water [adjusted to pH 7.2 with 4% (w/v) NaHCO₃ solution] and 17 ml. containing 24 units and 12.2 mg. of protein was obtained. Half of this was incubated in 0.05 *M*-tris buffer, pH 7.2, at 37° for 10 min. and the other half incubated with 100 μg . of each component of leucocidin in the same way. The suspensions were then centrifuged at 25000 *g* for 30 min. and the supernatant was treated with 0.4 ml. of 60% (w/v) HClO₄ solution at 0° for 25 min. The pellets were extracted with 2 ml. of 0.45 *N*-HClO₄ at 0° for 30 min. and centrifuged at 25000 *g* for 30 min. The total phosphorus and P_i contents of the acid-soluble fractions were determined. The acid-insoluble fraction of the pellet was extracted with 2 ml. of chloroform-methanol (2:1, v/v) at room temperature for 30 min. and centrifuged at 1500 *g* for 10 min. The extract contained the membrane lipids and their phosphorus content was determined. The material insoluble in chloroform-methanol contained the phosphoproteins and was washed with ether and dried in air. It was dissolved in 0.1 *N*-NaOH solution and the total phosphorus and protein content were determined.

Ultraviolet-absorption spectra. These were measured on the acid-soluble fractions of the membrane supernatant and pellet prepared in studying the distribution of phosphorus (see above).

Stability of membrane phosphorus. Samples of light membranes (3.0 units) were diluted to 5.0 ml. with ice-cold water immediately after preparation and centrifuged at 25000 *g* for 30 min. The pellets were then treated in one of two ways. (A) A 2 ml. portion of 0.45 *N*-HClO₄ was added at 0° to each of two pellets and one centrifuged immediately at 0° for 10 min. at 25000 *g*, and the total phosphorus and P_i were determined immediately on the supernatant. The other

pellet was extracted at room temperature for 75 min., centrifuged at 25000g for 10 min. at room temperature and the total phosphorus and P_i in the supernatant were determined. (B) Two pellets were suspended in 3 ml. of 0.15 M-NaCl-0.01 M-tris buffer, pH 7.2, and incubated at 37° for 10 min. They were centrifuged at 25000g for 20 min. at 0°. The supernatant was treated with 0.15 ml. of 9.2 N-HClO₄ at 0°, freed from precipitate by centrifuging at 25000g at 0° and the total phosphorus and P_i were determined. The insoluble parts of the two incubated membrane pellets were extracted with 2.0 ml. of 0.45 N-HClO₄, one at 0°, which was immediately centrifuged at 25000g for 10 min., and the other at room temperature, which was similarly centrifuged after standing for 75 min. The total phosphorus and P_i in the extracts from the insoluble parts of the incubated membrane pellets were determined.

Preparation of membranes from cells incubated with [³²P] P_i . Leucocytes (1×10^{10} – 3×10^{10} cells) were washed with phosphate-free Krebs-Ringer bicarbonate solution and suspended in 10 ml. of this solution. [³²P] P_i (0.2 ml. of 'PBS1' containing 100–400 μ C) was added and the cells were incubated at 37° for 30 min. They were then diluted with 200 ml. of ice-cold Krebs-Ringer phosphate solution centrifuged at 1500g for 5 min. and washed again in the same way. They were then suspended in 0.32 M-sucrose and light membranes were prepared. These were incubated alone or with added leucocidin and the distribution of radioactivity and phosphorus determined.

In a further experiment labelled membranes from 7.5×10^9 cells were prepared as described above and 3.0-ml. samples, containing 6.0 units, incubated alone or with added leucocidin for 10 min. at 37° in 0.05 M-tris buffer, pH 7.2. The mixtures were centrifuged at 25000g for 20 min. and the supernatants retained. The pellets were suspended ultrasonically in 0.5 ml. of 0.2% sodium deoxycholate-0.01 M-tris buffer, pH 7.2, or in 0.2% sodium deoxycholate-0.33 N-NH₃ solution. The suspensions of membrane were fractionated on columns containing 4 g. of Sephadex G-25 by using either of the above solutions as eluent. The effluent fractions (0.5 ml.) were analysed for radioactivity and ultraviolet absorption. The 'membrane peak', containing the material excluded from the gel, was obtained by combining the early fractions (elution volume 6–10 ml.) where the $E_{280\text{ m}\mu}^{1\text{ cm}}$ value was greater than 0.05. The 'intermediate fraction' (elution volume 10–14 ml.) was obtained by combining subsequent fractions containing less than 50 counts/min./fraction. The ' P_i peak' (elution volume 14–20 ml.) was then obtained by combining subsequent fractions until the radioactivity fell to less than 5 counts/min./fraction.

Adenosine-triphosphatase and phosphatase activities of membranes. To determine the concentration of membranes giving direct proportionality to activity, membranes were incubated with 1 mM-ATP (sodium salt)–1 mM-MgCl₂ in 1.0 ml. of 0.05 M-tris buffer, pH 8.0, for 10 min. at 37°, and 3.0 ml. of 0.45 N-HClO₄ was added at 0°. The suspensions were centrifuged at 1500g for 10 min. and the P_i in the supernatant was determined. The optimum pH was determined in the same way with mixtures containing 0.2 unit of membranes but with tris-maleic acid buffers in place of the tris-chloride buffer. To detect inhibition by strophanthin G this was included (final concn. 0.33 mM) with 0.2 unit of membranes in mixtures prepared and treated in the same way as in the determination of the optimum membrane

concentration and also in mixtures that contained in addition NaCl (20 mM) plus KCl (20 mM) or NaCl (50 mM) plus KCl (50 mM). The conditions in other experiments with membrane phosphatase are given in the Results section.

In assessing the sensitivity of the adenosine triphosphatase in deoxycholate-treated membranes to NaCl and KCl, ATP (tris salt) was used as substrate. To make this a column containing 5 ml. of Zeo-Karb 225 (H⁺ form) was washed with M-tris (free base) until the effluent was at pH 9 and then with water until the conductivity of the effluent was that of the water. Then a solution of 100 mg. of ATP (sodium salt) in 0.5 ml. of water was added to the column and washed through with 5 ml. of water. The concentration of the tris-ATP was determined from the ultraviolet absorption of the effluent. Tris-deoxycholate was prepared by passing 5 ml. of 1.2% (w/v) sodium deoxycholate solution through a similar column of Zeo-Karb 225 and washing it through with 2.5 ml. of water. The pH of the effluent solution was adjusted to 7 with M-tris (free base) and the concentration of the tris-deoxycholate determined by drying 2 ml. at 110°. To degrade the membranes with deoxycholate, light membranes (5.75 units) were treated with the amount of tris-deoxycholate indicated in Fig. 3 in 5 ml. of 0.5 M-tris buffer, pH 7.5, for 2 min. at 0° and centrifuged at 0° for 1 hr. at 40000 rev./min. (102000g) in the no. 40.2 rotor of the Spinco model L ultracentrifuge. The pellets were homogenized in 5 ml. of 0.25 M-sucrose and centrifuged again in the same way. The pellets were homogenized in 2.6 ml. of 0.25 M-sucrose and samples (0.1 ml.) incubated with 4 mM-MgCl₂–4 mM-tris-ATP–0.05 M-tris buffer, pH 8, for 10 min. at 37° alone or with NaCl (0.1 M) plus KCl (5 mM).

Incorporation of ³²P into light membranes. A sample of light membranes was treated with partially purified 'PBS1' and fractionated on Sephadex G-25 as described in Fig. 4. Fractions (0.5 ml.) eluted from the Sephadex were analysed for radioactivity and $E_{280\text{ m}\mu}$ was determined. The fractions with $E_{280\text{ m}\mu}$ greater than 0.1 were combined and the total radioactivity and $E_{280\text{ m}\mu}$ noted. Acetone (10 vol.) was added at 0° and the precipitate collected by centrifuging at 1500g for 15 min. The supernatant was added to the chloroform-methanol extract (see below) and the pellet extracted with 10 ml. of chloroform-methanol (2:1, v/v) for 20 min. at room temperature. The suspension was centrifuged at 1500g for 20 min. and the pellet extracted with 5 ml. of ether in the same way. The acetone, chloroform-methanol and ether extracts were combined and constitute the lipid fraction. The insoluble fraction was dried in air and extracted with 2 ml. of 10% (w/v) trichloroacetic acid at 0° for 30 min. The suspension was centrifuged at 16000g for 20 min. and the pellet extracted again with 2 ml. of 10% (w/v) trichloroacetic acid in the same way. The trichloroacetic acid extracts were combined and constitute the acid-soluble fraction. The acid-insoluble fraction is the phosphoprotein fraction and was dissolved in 1.0 ml. of 1.0 N-NaOH. The total phosphorus content and the radioactivity in the lipid, acid-soluble and phosphoprotein fractions were determined.

A further sample of light membranes was treated as described in Fig. 4 but the pellet was suspended ultrasonically in 0.2% sodium deoxycholate-0.3 N-NH₃ and eluted from Sephadex with this solvent.

The experiments to test ³²P incorporation into membranes from the highly purified 'PBS1' and [³²P]ATP were carried out as described in Fig. 4.

RESULTS

Composition of membranes. Analysis for nucleic acids was used during the purification of the material responsible for leucocidin elimination, to indicate contamination with nuclear material. It was found that mixed membranes contained less than 0.5 μ g. of RNA/mg. of membrane protein and less than 0.8 μ g. of DNA/mg. of membrane protein. Analysis for other compounds was done on light membranes.

Table 1 gives the gross composition of a sample of light membranes. The carbohydrate content is shown in Table 2. The composition of different samples has not varied more than 10% except with regard to the anthrone-positive material. These have differed threefold. In the separation on Sephadex G-25, anthrone-positive material was absent from the zone between the membrane and sucrose peaks but it is possible that some sucrose is occluded in the production of vesicles from the cell membrane and is not free to diffuse into the dextran gel. After incubation of light membranes (eluted from Sephadex) in 0.01 M-tris buffer, pH 7.5, at 37°

for 10 min. 11% of the anthrone-positive material and 18% of the *N*-acetylneuraminic acid were found in the membrane supernatant. The nucleotide content of light membranes is very small. If all the $E_{260\text{ m}\mu}$ value of the acid extract of light membranes were due to adenine less than 10 μ moles/mg. of membrane protein would be present.

The distribution of phosphorus in fractions of light membranes is shown in Table 3. The membranes were incubated at 37° for 10 min. and the supernatant and pellet analysed separately to provide comparison with membranes treated with leucocidin (see below). The phosphoprotein fraction of the pellets contained 42% of the protein present in the membrane preparation. Comparison of the phospholipid P/phosphoprotein P ratio of the material analysed in Table 3 (5.3) with that for the whole membrane suspension analysed in Table 1 (2.0) suggests that some of the phosphoprotein is liberated into solution during incubation of the membranes. The small amount of acid-soluble phosphorus in the membrane is also dependent on the treatment given to the membranes. Table 4 shows that there is an accumulation of P_i during extraction with acid and also a conversion of acid-soluble esterified phosphorus into P_i during incubation at 37°. Thus the P_i content of membranes shown in Table 3 is not to be taken as a constant feature of the membrane.

Effect of treatment with leucocidin on the composition of membranes. Incubation of membranes at 37° for 10 min. with 8 μ g. of each component of leucocidin/unit of membrane in 0.01 M-tris buffer, pH 7.2, or in 0.15 M-sodium chloride-0.01 M-tris buffer, pH 7.2, did not change the amount of protein, ultraviolet-absorbing material, anthrone-positive material or *N*-acetylneuraminic acid liberated into the supernatant. In two experiments the amount of P_i in the supernatant of 10 units of leucocidin-treated membranes was 1.4 μ g. and 0.2 μ g. respectively, greater than that in the supernatant of

Table 1. *Composition of light membranes*

Light membranes from 9×10^9 cells were dialysed against water [adjusted to pH 7.2 with 4% (w/v) NaHCO_3]. Samples of the dialysed suspension were taken for analysis.

	Amount present/ml.	Percentage of dry wt.
Dry wt.	4.05 mg.	
Dry wt. of non-lipid components	2.45 mg.	
Protein	1.95 mg.	48.5
Cholesterol	0.27 mg.	6.7
Lipid P	0.03 mg.	
Esterified fatty acid	1.38 μ equiv.	
Lipid N	< 0.05 mg.	
Total N	0.36 mg.	9
Phosphoprotein N	0.29 mg.	7.1
Phosphoprotein P	0.015 mg.	
$E_{260\text{ m}\mu}^{\text{lem.}}$ in 0.2% sodium deoxycholate-0.1 N-NaOH	3.35	

Table 2. *Carbohydrate content of light membranes*

A sample of light membranes (12 units in 0.5 ml.) was freed from sucrose on Sephadex G-25 and samples of the membrane fraction were analysed for protein, *N*-acetylneuraminic acid and anthrone-positive material. A further sample of light membranes (8 units) was treated in the same way and the protein, lipid-bound and lipid-free carbohydrate were determined.

	Total (μ moles/mg. of protein)	Lipid-bound (% of total)
<i>N</i> -Acetylneuraminic acid	0.018	30
Anthrone-positive material (as glucose)	1.3	5

Table 3. *Distribution of phosphorus in light membranes*

Light membranes were dialysed against water [adjusted to pH 7.2 with 4% (w/v) NaHCO_3] to give 8.5 ml., containing 12 units. This was incubated at 37° for 10 min. and centrifuged. The phosphorus distribution in the pellet and supernatant was determined.

Fraction	Distribution of P (μ g./10 mg. of membrane protein)
Supernatant P	8.6
Esterified P	9.5
Pellet P_i	4.0
Acid-soluble esterified P	8.2
Lipid P	130.0
Phosphoprotein P	24.1

Table 4. *Stability of bound phosphorus in membranes*

Samples containing 3 units of membranes were centrifuged and the pellets treated in two ways. (A) They were extracted with 0.45N-HClO₄ and the acid-soluble phosphorus was determined after 10 min. at 0° and after 85 min. at room temperature. (B) They were incubated at 37° for 10 min. in 0.15M-NaCl-0.01M-tris buffer, pH 7, and centrifuged. The soluble material was treated with 0.45N-HClO₄ and the soluble phosphorus determined after 10 min. at 0°. The insoluble parts of the incubated pellets were extracted with 0.45N-HClO₄ solution and the acid-soluble phosphorus was determined after 10 min. at 0° and after 85 min. at room temperature.

Material	Acid-soluble P ($\mu\text{g.}/10\text{mg.}$ of membrane protein)	
	P _i	Esterified P
A Pellet not incubated, analysed after 10 min. at 0°	0.56	27
Pellet not incubated, analysed after 85 min. at room temperature	3.45	27.8
B Pellet incubated, soluble fraction analysed	9.7	12.4
Pellet incubated, insoluble fraction analysed after 10 min. at 0°	2.25	4.5
Pellet incubated, insoluble fraction analysed after 85 min. at room temperature	2.78	8.3

normal membranes. Because of the variations in the acid-soluble phosphorus content of normal membranes (Table 4) it is improbable that these differences are significant.

The possibility that treatment with leucocidin changed the reactivity or amount of a very labile, metabolically active, phosphorus compound present in the membrane in a small amount was also investigated by preparing membranes from cells labelled with ³²P. Treatment of light membranes (5 units) with 100 $\mu\text{g.}$ of leucocidin for 10 min. at 37° did not change the amount or specific activity of the ³²P in the supernatant, acid-soluble, lipid or phospho-protein fractions. As there was evidence that phosphorus groups in the structural components of the membrane were labile to acid treatment, labelled membranes (6.0 units) were incubated alone or with leucocidin (100 $\mu\text{g.}$) at 37° for 10 min. and the pellet and supernatant separated. The pellets were suspended in deoxycholate solution (see Table 5) and fractionated on Sephadex G-25. The distribution of radioactivity in the fractions was not changed by leucocidin. Table 5 shows that there are present in the centrifuged membrane pellet phosphorus compounds that, in the presence of deoxycholate, can penetrate into the Sephadex particles. It is probable that these are occluded within the membrane vesicles and will depend on the amount in the cell suspension at the time of the homogenization. No significant difference was seen in the amount of occluded phosphorus in normal and leucocidin-treated membranes, which suggests that leucocidin does not change the permeability of the membrane to phosphorus, in accordance with the views of Woodin & Wieneke (1963).

Table 5 shows that some of the radioactivity bound to membranes from labelled cells is eluted by

Table 5. *Distribution of radioactivity in labelled membranes fractionated on Sephadex*

Light membranes (6.0 units in 3.0 ml. of 0.05M-tris buffer, pH 7.2) prepared from leucocytes labelled with ³²P were incubated at 37° for 10 min. and centrifuged. The supernatant was retained and the pellet suspended in 0.2% sodium deoxycholate-0.01M-tris buffer, pH 7.2, or in 0.2% sodium deoxycholate-0.33N-NH₃ solution and fractionated on Sephadex G-25. The total radioactivity in each fraction was determined.

Fraction	Total radioactivity (counts/min.)	
	Eluted at pH 7.2	Eluted with aq. NH ₃
Supernatant	4550	4250
Membrane peak	2200	866
Intermediate fraction	210	88
P _i peak	880	1788

0.33N-ammonia. It is probable that this radioactivity is derived from the impurity present in 'PBS 1' (see below).

Phosphatase activity in leucocyte membranes. With 1 mM-ATP (sodium salt) as substrate and magnesium chloride (1 mM), 0.058, 0.116 and 0.23 mg. of light-membrane protein liberated 3.5, 6.5 and 12.5 $\mu\text{g.}$ of P_i respectively and further characterization was done with 0.116 mg. of membrane protein. Fig. 1(a) shows the effect of Mg²⁺ and Ca²⁺ as activators and Fig. 1(b) the effect of variations of pH. The adenosine-triphosphatase activity did not vary with the Na⁺ or K⁺ concentration (Fig. 1c) and was not inhibited by strophanthin G (0.33 mM). Pre-treatment of the membranes with leucocidin (1 or 16 $\mu\text{g.}$ of each component/0.2 unit of membranes in 0.05M-tris buffer, pH 7.2) for 10 min. at 37° did not

Fig. 1. Adenosine-triphosphatase activity of light membranes. Mixtures (1.0 ml.) containing 0.2 membrane unit and ATP (1 mM) were incubated at 37° for 10 min. and the P_i was determined. (a) Effect of Mg^{2+} and Ca^{2+} . Mixtures contained 0.05 M-tris buffer, pH 8, MgCl_2 as indicated and EDTA (magnesium salt) (0.1 mM) or CaCl_2 as indicated and EDTA (calcium salt) (0.1 mM) or, where no MgCl_2 or CaCl_2 was present, EDTA (sodium salt) (0.1 mM). $\circ$, Concn. of Mg^{2+} ; $\blacksquare$, concn. of Ca^{2+} . (b) Effect of pH. Mixtures contained MgCl_2 (1 mM) and tris-maleic acid buffer (0.05 M) as indicated. (c) Effect of NaCl and KCl. Mixtures contained tris buffer, pH 8 (0.05 M), MgCl_2 (1 mM) and both NaCl and KCl as indicated.

Fig. 2. Effect of treatment with deoxycholate on the adenosine-triphosphatase activity of light membranes. Light membranes (5.75 units) were treated with tris-deoxycholate, as indicated, in 5 ml. of 0.5 M-tris buffer, pH 7.5, washed with 0.25 M-sucrose and suspended in 0.25 M-sucrose. Samples containing 0.22 membrane unit were tested for adenosine-triphosphatase activity with 4 mM-ATP (tris salt) as substrate, under the conditions described in Table 6. $\circ$, No NaCl or KCl present; $\bullet$, NaCl (100 mM) and KCl (5 mM) present.

affect the activity. When light membranes were treated with deoxycholate under the conditions used by Järnefelt (1964) with brain microsomes no sensitivity of the adenosine triphosphatase in the membranes to Na^+ or K^+ could be detected (Fig. 2).

Hydrolysis of phosphorus compounds by light membranes was not confined to ATP or ADP. Table 6 shows that glycerophosphate and AMP are also hydrolysed slowly. The activity of the membranes with glycerophosphate as substrate was decreased 2.5-fold when the membranes were pre-treated with deoxycholate (1 mg./ml.) under the conditions described in Fig. 2.

Incorporation of ^{32}P into light membranes. A labile phosphorus compound in a membrane could be a site of control of permeability. Woodin & Wieneke (1964) suggested that cross-linking of phosphate groups by Ca^{2+} contributes to the mechanical strength of membranes and that this is diminished during protein secretion. The possibility that there is a rapid exchange of membrane phosphorus with added P_i was therefore investigated.

Fig. 3 shows that, after incubating light membranes with partially purified 'PBS1' and subsequent fractionation on Sephadex G-25 with deoxycholate, 1% of the added radioactivity is found in the membrane peak. The acid-soluble, lipid and phosphoprotein fractions contained 78, 2 and 20% of the radioactivity respectively. The uptake of radioactivity was also found if membranes were treated with partially purified 'PBS1' at 0° and if the unlabelled P_i concentration was increased from 1 to 20 mM only a threefold decrease in the amount of radioactivity associated with the membranes was found. The uptake of radioactivity by membranes under these conditions appears to be due to an impurity that survives the purification procedure of

Table 6. *Phosphatase activity of light membranes*

Mixtures (1.0 ml. volume) containing tris buffer, pH 8 (0.05 M), membranes as indicated, substrate (4 mM) and MgCl_2 (4 mM) were incubated at 37° for 10 min. and the P_i was determined.

Membrane protein (mg.)	Substrate ...	P_i liberated (μmole)			
		ATP	ADP	AMP	3-Glycerophosphate
0.04		0.135	0.07	0.01	0.01
0.09		0.26	0.14	0.02	0.013
0.18		0.47	0.29	0.025	0.03

Fig. 3. Elution of membranes containing ^{32}P from Sephadex G-25. Light membranes (12.75 units) were incubated in 1.9 ml. of 2 mM-EDTA (sodium salt)-5 mM-phosphate buffer-0.05 M-tris buffer, pH 7.2, with partially purified 'PBS1' (8×10^6 counts/min.) for 10 min. at 37° and diluted with 4 ml. of water at 0°. The mixtures were centrifuged at 25000g for 15 min. and the pellets suspended ultrasonically in 0.5 ml. of 0.2% sodium deoxycholate-0.1 M-phosphate buffer, pH 7.2. The suspension was eluted from a column containing 4 g. of Sephadex G-25 with 0.2% sodium deoxycholate solution. The radioactivity (○) and $E_{280 \text{ m}\mu}$ (Δ) were determined in the eluted fractions.

Suelter *et al.* (1961). When the membranes were labelled with partially purified 'PBS1' and fractionated on Sephadex as described in Fig. 3 no radioactivity was found in the membrane peak if 0.2% sodium deoxycholate-0.33 N-ammonia solution was used as the eluent. The presence of an alkali-labile component in membranes prepared from leucocytes labelled with 'PBS1' is recorded above.

Highly purified 'PBS1' failed to label membranes when 5×10^6 counts/min. were added to 2.3 units of

light membranes in 0.3 ml. of 1 mM-phosphate-0.05 M-tris buffer, pH 7.2, and incubated at 37° for 10 min. alone or with 50 μg . of leucocidin when none or any of the following compounds was present (final concentrations are given): EDTA (sodium salt) (0.1 mM); magnesium chloride (1 mM); calcium chloride (1 mM); ATP (magnesium salt) (1 mM); ATP (calcium salt) (1 mM). When 1 mM- ^{32}P ATP (10^6 counts/min.) was substituted for the highly purified 'PBS1', 10^3 counts/min. was found associated with the membranes. The same uptake of radioactivity was found with 5 mM- ^{32}P ATP (10^6 counts/min.) and it is due to the impurity in the 'PBS1' used to prepare the ^{32}P ATP. Glynn & Chappell (1964) report the presence of 2% of impurity in their ^{32}P ATP preparation.

Oxidative enzymes in the membrane fraction. The NADH_2 -cytochrome *c*-oxidoreductase activity of light membranes was 12.2 units/mg. of protein. The NADPH_2 -cytochrome *c*-oxidoreductase activity of light membranes was less than 0.8 unit/mg. of protein. Direct oxidation of NADH_2 or NADPH_2 by the membranes alone or in the presence of 0.3 mM-menadione sodium bisulphite could not be detected. Evans & Karnovsky (1961) described the presence of enzymes catalysing the oxidation of NADH_2 by oxygen in the leucocyte but the localization in the cell is not clear (Cagan & Karnovsky, 1964). (The effect of menadione sodium bisulphite was investigated as it can simulate some of the effects of phagocytosis in the leucocyte.)

DISCUSSION

The light-membrane fraction contains 48–60% of protein and the remainder is lipid. There is less than 2 equiv. of esterified fatty acid/equiv. of lipid phosphorus and so triglycerides cannot be present to any extent. The phospholipid/cholesterol molar ratio is 1.4. This differs from the ratio for the total leucocyte lipids but resembles that for the lipids of the erythrocyte (Willmer, 1961). Labile phosphorus compounds are present in the membrane but their analysis is complicated by phosphorus that is probably occluded in the vesicles. The amount of P_i appearing to be in the membrane is also influenced

by incubation and by acid extraction and the identification of the labile phosphorus compounds will require additional analytical methods to those employed here. When membranes are treated with leucocidin the composition is not detectably changed. This is consistent with the observation of Woodin (1961) that the structural components of the leucocyte are not degraded during treatment with leucocidin.

One consequence of leucocidin poisoning is a change in the phosphorus metabolism of the cell. The adenosine-triphosphatase activity increases (Woodin, 1961) and the rate at which nucleotides turn over decreases. In the presence of Ca^{2+} a new P_i -nucleotide exchange reaction appears at the cell surface (Woodin & Wieneke, 1963). All of these effects are observed in the first few minutes of poisoning and if they resulted directly from leucocidin action there should be some manifestation of altered phosphorus metabolism at the site of action of leucocidin. The results in this paper give no evidence for a change in amount of reactivity of a phosphorus compound or phosphatase after the interaction of leucocidin with the isolated cell membrane. The failure to find a chemical change induced in the membrane is consistent with the conclusion of Woodin & Wieneke (1966) that the primary action of leucocidin is to induce a steric change in the membrane. It is probable that the changes in phosphorus metabolism follow from this with the participation of the cytoplasmic components.

The presence of phosphorus compounds other than P_i in commercial $[\text{P}^{32}]\text{P}_i$ has been noted by other authors (Suelter *et al.* 1961). The observations in the present paper show that membrane fractions have a considerable avidity for such contaminants and particular care is required in the purification of phosphorus used to label isolated membranes or even membrane fractions present in the intact cell.

It has been stated in the introduction to this paper that the light-membrane fraction may contain fragments from the nuclear membrane and the Golgi apparatus. It is not possible to assert that the enzyme activities observed in the membrane fraction are present in the cell surface membrane. However, the light-membrane fraction does contain material derived from the cell surface, and the adenosine triphosphatase does not show a dependence on Na^+ or K^+ . The studies of Elsbach & Schwartz (1959) show that the electrolyte balance in the leucocyte differs from that in other cells.

Woodin & Wieneke (1966) found that the material that inactivates leucocidin is present in the granule fraction prepared by the method of Cohn & Hirsch (1960a), but if the membranes are removed by flotation the insoluble fraction of the cell homogenate, which contains the granules, does not do this.

It is probable that the granule fraction prepared by the method of Cohn & Hirsch (1960a) contains fragments of the cell membrane. Some of the alkaline phosphatase in the granule fraction may be due to this contamination. Cohn & Hirsch (1960b) record that the alkaline phosphatase of the granules was not completely released into the cytoplasm after phagocytosis. Woodin (1962) found that the alkaline phosphatase of the leucocyte was not released into the medium of the leucocidin-treated cell to the same extent as the other granule enzymes.

The significance of oxidative enzymes in the membrane fraction is not known; it is not known if the mature leucocyte contains cytochrome c. However, changes in oxidative properties of the leucocyte occur during phagocytosis (Karnovsky, 1962) and these have frequently been ascribed to alterations in the cytoplasmic granules. It is possible that some of the changes may result from alterations in the cell membrane.

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