The Polyphosphoinositide Content of the Leucocyte, Erythrocyte and Macrophage

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1. The polyphosphoinositide content of macrophages and the cell membranes of leucocytes and erythrocytes was determined by an extension of the 'acid-hydrolysis' procedure of Dawson & Eichberg (1965). The estimation was controlled by adding a little highly radioactive polyphosphoinositide to the tissue extracts before fractionation. Several standard methods for determining polyphosphoinositides gave low recoveries when applied to leucocytes, and it is suggested that these cells contain materials that form complexes with the polyphosphoinositides and interfere with the assay. 2. The method for the preparation of leucocyte cell surface membranes has been modified.

Woodin & Wieneke (1967) found that triphosphoinositide from ox brain inactivated the two components of leucocidin and suggested that this is the basis of the inactivation of leucocidin by the leucocyte cell membrane. The presence of polyphosphoinositides in leucocytes from peritoneal exudates was inferred by Sastry & Hokin (1966) because radioactive material isolated from extracts of leucocytes labelled with ^{32}P behaved chromatographically in the same way as authentic triphosphoinositide. Quantitative data on the polyphosphoinositide content of leucocytes are presented in the present paper. Erythrocytes and macrophages frequently contaminate polymorphonuclear leucocytes from peritoneal exudates and we have determined the polyphosphoinositide content of these cells too.

In the present work the estimation of the polyphosphoinositides was controlled by adding a little highly radioactive polyphosphoinositide to the tissue extract and the subsequent estimation was by an extension of the 'acid-hydrolysis' procedure of Dawson & Eichberg (1965). In preliminary experiments, in which several methods were used to estimate the polyphosphoinositide content of leucocytes, very low recoveries were obtained. This suggests that reports of the absence of triphosphoinositide from tissues (cf. Prottey & Hawthorne, 1966), tested by methods not controlled in this way, must be treated with reserve.

METHODS

Materials. Polymorphonuclear leucocytes (called leucocytes in this paper) were obtained from peritoneal exudates of rabbits as described by Woodin (1961). To obtain rabbit

macrophages and erythrocytes 50 ml. of liquid paraffin was injected into the peritoneal cavity; after 4 days the animals were anaesthetized with ether and their throats cut. Blood was collected in a beaker containing 2 mg. of heparin and the erythrocytes were recovered by centrifuging at 3000g for 20 min. They were washed three times with 6 vol. of 0.145 M-NaCl solution by centrifuging in the same way. The macrophages were washed out of the peritoneal cavity with 250 ml. of 0.145 M-NaCl solution. The washings were cooled to 0° and centrifuged at 3000g for 5 min. at 0°. The pellets were extracted with chloroform-methanol (1:1, v/v) and stored in this medium at 0° until sufficient cells had accumulated to prepare the lipid extract.

Erythrocyte membranes. These were obtained by pouring the washed erythrocytes into 6 vol. of ice-cold 5 mM-tris buffer, pH 7.2, which was vigorously stirred. After 10 min. the mixture was centrifuged at 3000g for 30 min. The pellet was washed by centrifuging in the same way at 0° with 5 mM-tris buffer, pH 7.2, until the supernatant was colourless. The erythrocyte 'ghosts' were then gently stirred with a little 5 mM-tris buffer, pH 7.2, to remove them from a tightly packed pellet of red material and added to 10 vol. of chloroform-methanol (1:1, v/v).

Leucocyte membrane fractions. To minimize the amount of multivalent cations in the leucocyte membranes before determination of the polyphosphoinositides, EDTA (final concn. 2 mM) was added to the leucocytes in ascitic fluid and EDTA (final concn. 1 mM) to the sucrose solutions used in the preparation of the 'light membranes' described by Woodin & Wieneke (1966a). When this was done a double band was obtained at the interface between the 30% and 44% (w/v) sucrose solutions. The material in the bands was separated by centrifuging in a 40–44% (w/v) sucrose gradient, and that in the lighter band inactivated leucocidin at a lower concentration than the heavier. The light fraction was therefore relatively richer in material derived from the cell surface membrane. To obtain leucocyte membranes for polyphosphoinositide determination leucocytes in ascitic fluid were treated with 2 mM-EDTA and

washed and homogenized in 11.6% (w/v) sucrose solution containing EDTA (1mM) as described by Woodin & Wieneke (1966a). The homogenate (6ml./tube) was put on top of a discontinuous density gradient consisting of layers (8ml. each) of 30, 40 and 50% (w/v) sucrose all containing EDTA (1mM) and centrifuged at 30000 rev./min. (78480 g_{av}) for 1 hr. in the no. 30 rotor of the Spinco model L ultracentrifuge. The material at the interface between the 30% and 40% (w/v) sucrose solutions is referred to as the leucocyte surface-membrane fraction, that at the interface between the 40 and 50% (w/v) sucrose solutions the leucocyte cytoplasmic-membrane fraction, and the material in the pellet the leucocyte cell-residue fraction. The identification of the surface-membrane fraction was based on the inactivation of leucocidin in the same way as the leucocyte (Woodin & Wieneke, 1966a). The first two fractions were concentrated by adding 1mM-tris buffer, pH 7.2, to lower the sucrose content to about 30% (w/v) and centrifuging the suspension on top of 0.5 ml. of 50% (w/v) sucrose solution at 105000 g for 1 hr.

Preparation of lipid extracts. (a) 'Neutral extract' of lipids. This was prepared by extracting material twice with 10 vol. of chloroform-methanol (1:1, v/v) and then twice with 10 vol. of chloroform-methanol (2:1, v/v). The chloroform content of the combined extracts was increased to twice the methanol content and the mixtures were shaken with 0.2 vol. of *N*-HCl. The lower layer was shaken with 2 vol. of chloroform-methanol-*N*-HCl (3:48:47, by vol.) and then twice with 2 vol. of chloroform-methanol-5mM- $CaCl_2$ (3:48:47, by vol.). The final lower layer was dried and dissolved in chloroform.

(b) Chloroform-methanol-HCl extract. This was prepared by extracting the residues resulting from the preparation of the neutral extract four times with 10 vol. of chloroform-methanol-conc. HCl (800:400:3, by vol.) at 37° for 20 min. After centrifuging at 3000 g for 10 min. the extracts were filtered through a sintered-glass filter and used for determination of the polyphosphoinositides.

Determination of the polyphosphoinositides in acid extracts of lipids. The phosphorus content of the extracts was determined and radioactive triphosphoinositide and radioactive diphosphoinositide (2000–8000 counts/min. of each) were added to the remainder. The extracts were shaken with 0.2 vol. of *N*-HCl and centrifuged. The lower layer was separated from the upper layer and the interface. The lower layer (containing 70–80% of the added radioactivity) was dried in a tube on a rotary evaporator at about 10°. The dry material was dissolved in 5 ml. of chloroform and neutralized with NH_3 gas. The solution was dried and the solid boiled with 5 ml. of acetone for 2 min., dried, boiled again with 5 ml. of ethanol for 2 min. and dried. The solid was extracted six times with 0.5 ml. of chloroform-methanol-conc. HCl (800:400:3, by vol.) and the extracts were filtered through a small sintered-glass filter into a calibrated tube. The extracts obtained from leucocytes and macrophages were shaken with 0.2 vol. of 5mM- $CaCl_2$ and then four times with 3 vol. of chloroform-methanol-5mM- $CaCl_2$ (3:48:47, by vol.) to give the calcium salts of the polyphosphoinositides. The volume of the lower layer was reduced to about 1 ml. by this procedure. When the erythrocyte extracts were treated in this way an interfacial precipitate that contained 50–60% of the radioactivity was produced on shaking with 5mM- $CaCl_2$. To prevent this loss the extracts of erythrocytes were first shaken with 0.2 vol.

of *N*-HCl and then with 3 vol. of chloroform-methanol-*N*-HCl (3:48:47, by vol.), the interfacial precipitate being rejected each time. The polyphosphoinositides in the lower layer were then converted into the calcium salts as described above. Leucocyte and erythrocyte extracts were now treated identically and diluted tenfold with chloroform to decrease the methanol concentration. The solutions were applied to a column containing 5 mg. of silicic acid/ μg . of lipid P in the original extract. Impurities were removed by washing with 20 ml. of chloroform, and the polyphosphoinositides (as calcium salts) were eluted with 100 ml. of chloroform-methanol (9:1, v/v) and 100 ml. of chloroform-methanol-water (75:25:2, by vol.). The eluates were dried on a rotary evaporator and the solid was dissolved in 4 ml. of chloroform-methanol-conc. HCl (800:400:3, by vol.). Calcium was now removed by shaking the solution with 0.2 vol. of *N*-HCl and then four times with 3 vol. of chloroform-methanol-*N*-HCl (3:48:47, by vol.). The final lower layer was dried and the solid hydrolysed in 0.5 ml. of 5.8 *N*-HCl at 100° for 10 min. The hydrolysate was shaken with 5 ml. of chloroform and then 5 ml. of ether. It was then diluted with 0.5 ml. of water and passed down a column (35 cm. $\times$ 0.5 cm.) containing Zeo-Karb 225 (The Permutit Co., London, W. 4) suspended in *N*-HCl. The column of Zeo-Karb 225 was washed for 8 hr. with 5.8 *N*-HCl before use. Fractions from the column were collected in flat plastic dishes, and those containing the radioactivity were combined and dried *in vacuo*. Water (1 ml.) was added to the solid and excess of NH_3 gas was passed in. The radioactivity in solution was determined, and the solution was concentrated *in vacuo* to about 0.05 ml. and applied to washed paper (Whatman no. 1; 46 cm. $\times$ 57 cm.). The extent of recovery of the radioactivity was determined by counting the hydrolysate on the paper, and the polyphosphoinositide hydrolysis products were separated by chromatography and ionophoresis at pH 4.2 (Dawson & Eichberg, 1965). The paper was dried and cut in strips (2 cm. $\times$ 0.5 cm.) and the radioactivity determined. The strips containing inositol diphosphate and inositol triphosphate respectively and the origin of the chromatogram were digested in 1 ml. of 72% (w/v) $HClO_4$ and the phosphorus was determined. The radioactivity in the 2-methylpropan-1-ol extracts obtained in the phosphorus estimation was also measured.

Determination of the polyphosphoinositides in neutral extracts of lipids. To the calcium salts of the lipids in the neutral extract radioactive triphosphoinositide and radioactive diphosphoinositide (2000–8000 counts/min. of each) were added and the mixtures dried. The solid was boiled for 2 min. with 10 ml. of acetone, dried, boiled again with 10 ml. of ethanol and dried. The solid was extracted with six 0.05 ml. lots of chloroform-methanol-conc. HCl (800:400:3, by vol.). The lipids in the extract were converted into the calcium salts in preparation for silicic acid chromatography and treated subsequently in the same way as the lipids in the chloroform-methanol-HCl extract, except that after the ether wash the acid hydrolysate (1 ml.) was shaken with 4 ml. of chloroform-methanol (2:1, v/v). The extracted hydrolysate was concentrated to about 0.5 ml., and the acid content determined by titration and adjusted to 3.0 *N* before the hydrolysate was added to the Zeo-Karb 225.

Preparation of radioactive polyphosphoinositides. Four rabbit brains were sliced and incubated for 90 min. at 38° in

300 ml. of phosphate-free Krebs-Ringer bicarbonate solution (Umbreit, Burris & Stauffer, 1957) containing 10 mc of [32 P]orthophosphate and 200 mg. of glucose. The suspensions were then cooled to 0° and centrifuged at 0° at 3000g for 10 min. The supernatant was poured off and the sediment treated with 10 vol. of chloroform-methanol (1:1, v/v) at room temperature. The insoluble fraction was filtered off and treated twice with 10 vol. of chloroform-methanol (1:1, v/v) and twice with 10 vol. of chloroform-methanol (2:1, v/v). The chloroform-methanol-HCl-soluble lipids were then prepared as described above. The polyphosphoinositides were partially purified by the method of Dittmer & Dawson (1961), except that the precipitation of the triphosphoinositide from chloroform solution with methanol was not carried out but the acid extract obtained from the acetone- and ethanol-insoluble material was converted into the calcium salt and fractionated on silicic acid as described above. The polyphosphoinositides in the eluate from the silicic acid column were freed from calcium by treatment with N-HCl and then converted into the ammonium salts. They were applied to formaldehyde-treated paper (Hörhammer, Wagner & Richter, 1959) and separated by ascending chromatography in the upper phase of butanol-acetic acid-water-ether (20:5:25:6, by vol.) for 48 hr. A pad of paper was attached to the chromatogram 25 cm. from the origin. The positions of the triphosphoinositide and diphosphoinositide were determined by assaying the radioactivity on the paper and they were eluted with chloroform-methanol-conc. HCl (800:400:3, by vol.). They were then converted into the ammonium salts. On hydrolysis with carrier polyphosphoinositides and separation of the resulting inositol phosphates by ionophoresis (Dawson & Eichberg, 1965) less than 2% of the radioactivity from diphosphoinositide was present in the spot occupied by inositol triphosphate, but of the radioactivity in triphosphoinositide 20% was recovered in the spot occupied by inositol diphosphate. When the radioactive diphosphoinositide was chromatographed on formaldehyde-treated paper with carrier polyphosphoinositide 10% of the radioactivity was found in the position occupied by triphosphoinositide. This suggested that under the conditions used here multiple spots were produced and on formaldehyde-treated paper some of the diphosphoinositide was present in the position occupied by the triphosphoinositide. In determining the polyphosphoinositide content of cell extracts from the recovery of added radioactive polyphosphoinositide corrections were made for the presence of radioactive diphosphoinositide in the labelled triphosphoinositide preparation. Typical specific activities of the labelled triphosphoinositide and labelled diphosphoinositide were 4400 counts/min./ μ g. of P and 18000 counts/min./ μ g. of P respectively.

Other methods. Inositol, protein, phosphorus, radioactivity and leucocidin activity were determined as described by Woodin & Wieneke (1967). To determine the inactivation of leucocidin by leucocyte membranes these were washed by centrifuging with an equal volume of 1 mM-tris buffer, pH 7.2, at 30000g for 20 min. and suspended in this buffer. The inactivation of leucocidin was determined as described in Fig. 1 and samples of the membranes were also analysed for protein (with bovine serum albumin as standard) and phospholipid phosphorus, to enable comparison with the ability of erythrocyte membranes to inactivate leucocidin, and with the data of Woodin & Wieneke (1966a).

RESULTS

Preliminary observations on the estimation of polyphosphoinositides in leucocytes. Four methods are available for the small-scale separation and estimation of polyphosphoinositides: separation of the inositol phosphates after acid hydrolysis (Dawson & Eichberg, 1965), chromatography of the polyphosphoinositides on formaldehyde-treated paper (Hörhammer *et al.* 1959), separation of the glycerol inositol phosphates after alkaline hydrolysis (Hendrickson & Ballou, 1964) and chromatography of the polyphosphoinositides on silicic acid-impregnated paper (Santiago-Calvo, Mulé, Redman, Hokin & Hokin, 1964). When a sample of radioactive polyphosphoinositides from brain (13 μ g. of P; 1200 counts/min. of triphosphoinositide and 2000 counts/min. of diphosphoinositide) was chromatographed on silicic acid-impregnated paper (Whatman SG 81) under the conditions of Santiago-Calvo *et al.* (1964) the radioactivity remained within 1 cm. of the origin and the two polyphosphoinositides were not separated. This procedure probably is critically dependent on the nature of the silicic acid-impregnated paper. The other three methods gave good separation of the polyphosphoinositides and good recovery of the phosphorus and radioactivity. However, preliminary experiments indicated that the methods could not be applied directly to leucocyte extracts.

The presence of some polyphosphoinositide in leucocytes was shown by labelling 5×10^9 leucocytes in 100 ml. of phosphate-free Krebs-Ringer bicarbonate solution (Umbreit *et al.* 1957) for 1 hr. with 8×10^9 counts/min. of [32 P]orthophosphate. A highly radioactive chloroform-methanol-hydrochloric acid extract was obtained, and, when this was mixed with 1 mg. of purified polyphosphoinositide from brain and the procedure of Dawson & Eichberg (1965) for the isolation of the inositol phosphates applied, the region on the chromatogram containing inositol triphosphate contained 12000 counts/min. of radioactivity. However, when the procedure of Dawson & Eichberg (1965) was applied directly to 10^{10} leucocytes less than 1 μ g. of inositol could be recovered from the final chromatogram in the position corresponding to inositol triphosphate.

In a further experiment 2×10^{10} cells were labelled with [32 P]orthophosphate as described above and the chloroform-methanol-hydrochloric acid extract was prepared. This was fractionated with water (0.2 vol.) and the lower layer dried. The solid was boiled with acetone and then with ethanol, dried and extracted with chloroform-methanol-conc. hydrochloric acid (800:400:3, by vol.). The extract was fractionated with 0.2 vol. of water and the lower layer was dried, dissolved in chloroform

and neutralized with methanolic sodium hydroxide. The product contained 5×10^3 counts/min., but when chromatographed on formaldehyde-treated paper all the radioactivity remained at the origin.

The alkaline-hydrolysis procedure also could not be applied directly to leucocyte extracts. The chloroform-methanol-hydrochloric acid extract was prepared from a sample of leucocyte surface membrane containing 30 $\mu\text{g.}$ of total phospholipid P and added to 500 $\mu\text{g.}$ of purified triphosphoinositide

and 300 $\mu\text{g.}$ of purified diphosphoinositide. The mixture was treated with 0.2 vol. of *N*-hydrochloric acid and the lower layer dried. The solid was deacylated and the product chromatographed as described by Hendrickson & Ballou (1964). Only 38% of the triphosphoinositide and 15% of the diphosphoinositide were recovered as the corresponding glycerol inositol phosphates.

It was thus apparent that polyphosphoinositides were present in leucocytes but that their estimation would require modification of the methods available. The procedure used in the present work is an extension of the acid-hydrolysis method of Dawson & Eichberg (1965).

Quantitative estimation of polyphosphoinositides. Table 1 gives the recovery of radioactive polyphosphoinositides added to the chloroform-methanol-hydrochloric acid extract of erythrocyte membranes and fractionated as described above. A similar overall recovery was obtained with material derived from leucocytes. The bulk of the polyphosphoinositides in the chloroform-methanol-hydrochloric acid extract of leucocytes could be transferred to the upper layer by repeated extraction with 3 vol. of chloroform-methanol-*N*-hydrochloric acid (3:48:47, by vol.), but after the dried extract had been boiled with acetone and with ethanol this no longer occurred. Similar recoveries of labelled triphosphoinositide and labelled diphosphoinositide were obtained in the acid hydrolysate when they were added respectively to two samples of chloroform-methanol-hydrochloric acid extracts of leucocyte cell residues, but subsequent manipulations gave different recoveries of the two inositides. In

Table 1. *Distribution of radioactive polyphosphoinositides fractionated together with the chloroform-methanol-hydrochloric acid extract from erythrocytes*

The chloroform-methanol-HCl extract from erythrocytes was mixed with ^{32}P -labelled triphosphoinositide (2.16 $\mu\text{g.}$ of P; 3200 counts/min.) and ^{32}P -labelled diphosphoinositide (0.86 $\mu\text{g.}$ of P; 3700 counts/min.). The mixture was treated successively with *N*-HCl by the Folch procedure, hot acetone and hot ethanol, *N*-HCl by the Folch procedure again and silicic acid chromatography, and then hydrolysed in 5.8*N*-HCl. The hydrolysate was treated with Zeo-Karb 225 to remove cations.

Material	Radioactivity present (% of amount added)
Acid-soluble lipids	100
First <i>N</i> -HCl treatment, upper layer	21
Second <i>N</i> -HCl treatment, upper layer	2.7
Silicic acid, unadsorbed material	0.4
Silicic acid, eluted fraction	50
Hydrolysate after Zeo-Karb treatment	43

Table 2. *Polyphosphoinositide content of the chloroform-methanol-hydrochloric acid extracts of leucocytes, erythrocytes and macrophages*

The polyphosphoinositides were estimated as the inositol phosphates after purification of the acid chloroform-methanol-HCl extract in the presence of added radioactive polyphosphoinositides.

Cell fraction	Total phospholipid content of cell fraction (mg. of P)	Labelled triphosphoinositide added ($\mu\text{g.}$ of P)	Labelled diphosphoinositide added ($\mu\text{g.}$ of P)	Labelled triphosphoinositide recovered (%)	Labelled diphosphoinositide recovered (%)	Triphosphoinositide in cell fraction (m-moles/g. atom of lipid P)	Diphosphoinositide in cell fraction (m-moles/g. atom of lipid P)
Leucocyte surface membrane	2.6	2.1	0.7	20	25	2.7	1.0
Leucocyte cytoplasmic membrane	3.2	5.4	2.2	19	31	2.8	0.96
Leucocyte cell residue	2.8	3.9	5.1	18	25	1.2	1.3
Erythrocyte cell membrane	4.7	2.2	0.86	21	38	5.3	1.6
Macrophage whole cell	1.8	5.8	2.8	17	33	4.0	4.0

Table 3. *Polyphosphoinositide content of the neutral extract of lipids from leucocytes and erythrocytes*

The polyphosphoinositides were estimated as the inositol phosphates after purification of the neutral extract of lipids in the presence of added radioactive polyphosphoinositides.

Cell fraction	Total phospholipid content of cell fraction (mg. of P)	Labelled triphosphoinositide added (μ g. of P)	Labelled diphosphoinositide added (μ g. of P)	Labelled triphosphoinositide recovered (%)	Labelled diphosphoinositide recovered (%)	Triphosphoinositide in cell fraction (m-moles/g. atom of lipid P)	Diphosphoinositide in cell fraction (m-moles/g. atom of lipid P)
Leucocyte surface membrane	2.6	5.1	3.0	15	30	3.6	11
Leucocyte cytoplasmic membrane	3.2	5.4	3.0	7	28	2.0	5.7
Leucocyte cell residue	2.8	5.4	3.0	15	29	1.0	2.8
Erythrocyte membrane	4.7	—	3.0	—	28	—	2.8

particular, when a hydrolysate from leucocyte surface membranes, with added radioactive triphosphoinositide and diphosphoinositide, was dried without treatment with Zeo-Karb only 50% of the radioactivity could be dissolved to transfer to paper, and of that added to paper 60% remained at the origin after chromatography and ionophoresis. Most of the radioactivity found in the position occupied by the inositol phosphates was derived from diphosphoinositide; the inositol triphosphate remained at the origin. In a separate experiment it was found that $^{45}\text{Ca}^{2+}$ added to crude ox brain polyphosphoinositides in chloroform-methanol-conc. hydrochloric acid (800:400:3, by vol.) could be removed by fractionation with 0.2 vol. of *N*-hydrochloric acid followed by three 3 vol. lots of chloroform-methanol-*N*-hydrochloric acid (3:48:47, by vol.). This suggested that the poor recovery of inositol triphosphate in acid hydrolysates of tissue extracts was due to complex-formation with cationic material liberated during the acid hydrolysis. The inositol phosphates in a sample of acid hydrolysate from leucocyte surface membranes were recovered in only 30% yield when added to Zeo-Karb 225 (H^+ form) suspended in water, but if the resin was thoroughly washed with 5.8*N*-hydrochloric acid and used suspended in *N*-hydrochloric acid recovery was quantitative. Presumably under these conditions the ionization of the inositol phosphates is sufficiently suppressed to prevent complex-formation with multivalent cationic material retained by the resin. It was found that with a column (35 cm. $\times$ 0.5 cm.) of Zeo-Karb 225 (H^+ form) 2.5 μ moles of arginine and 0.5 μ g. ion of $^{45}\text{Ca}^{2+}$ were not eluted by 20 ml. of *N*-hydrochloric acid.

Hydrolysate treated with Zeo-Karb 225 could be

transferred to paper with 80% recovery, and of the radioactivity put on paper only 5–8% remained at the origin. A corresponding increase in the recovery of inositol triphosphate was found.

Tables 2 and 3 give the polyphosphoinositide content of leucocytes, erythrocytes and macrophages. The results are expressed in terms of the total lipid phosphorus content, as the protein and dry weight can be changed by washing. Representative values for the lipid P/protein ratios are 2.2 μ g. of lipid P/100 μ g. of protein for the leucocyte surface membrane and 1.75 μ g. of lipid P/100 μ g. of protein for the leucocyte cytoplasmic membrane. If the membranes used in the present work have the same phospholipid content as the surface-membrane fraction studied by Woodin & Wieneke (1966b), and if the molecular weight of triphosphoinositide is taken as 900, the triphosphoinositide content of the leucocyte surface membrane will be 0.15% of the dry weight.

Similar amounts of triphosphoinositide are found in the leucocyte, erythrocyte and macrophage, and as the leucocyte preparations used in the present work never contained more than 5% of erythrocytes or macrophages the triphosphoinositide found in the leucocyte fractions is not due to contamination from this source.

Inactivation of leucocidin by membranes derived from leucocytes and erythrocytes. Fig. 1 shows the inactivation of leucocidin by leucocyte membrane fractions. The activity associated with the cytoplasmic-membrane fraction may be due to contamination by surface membrane. An amount of erythrocyte membrane containing 50 μ g. of phospholipid P failed to inactivate 0.6 μ g. of either component of leucocidin when both were present under the conditions used in Fig. 1.

Fig. 1. Inactivation of leucocidin by leucocyte membranes. Leucocyte membranes were maintained with $0.6 \mu\text{g.}$ of one or both components of leucocidin in 1.0 ml. of 0.145 M-NaCl in 0.05 M-tris buffer, $\text{pH } 7.2$, for 30 min. at room temperature and then 0.05 ml. of 2% (w/v) bovine serum albumin in water was added. The mixtures were centrifuged at $30\,000g$ for 30 min. and the leucocidin activity in the supernatants was determined. ○, Surface membrane with both components of leucocidin, S component activity; △, surface membrane with both components of leucocidin, F component activity; ●, surface membrane with S component of leucocidin only; ▲, surface membrane with F component of leucocidin only; ⊙, cytoplasmic membrane with both components of leucocidin, S component activity; ▲, cytoplasmic membrane with both components of leucocidin, F component activity.

DISCUSSION

The results presented in this paper do not give a precise value for the triphosphoinositide content of leucocytes, erythrocytes and macrophages, because it is not known how much triphosphoinositide is degraded to diphosphoinositide during the isolation of the membranes. The triphosphoinositide content of the leucocyte surface membrane will therefore be at least 6.3 m-moles/mole of phospholipid. The corresponding value for myelin is 14 m-moles/mole of phospholipid (Eichberg & Dawson, 1965). From Fig. 1 of the present paper $0.6 \mu\text{g.}$ of the S component of leucocidin can be inactivated by membranes containing $0.4\text{--}0.8 \mu\text{mole}$ of phospholipid, and these amounts will contain at least $2.5\text{--}5.0 \text{ m}\mu\text{moles}$ of triphosphoinositide. These values are similar to the amount of purified triphosphoinositide required to inactivate the S component of leucocidin (Woodin & Wieneke, 1967). Triphosphoinositide is a rare component of the leucocyte surface membrane, and impedance measurements (Woodin, 1966) indicate that the sites of action of leucocidin are few in number.

There is no correlation between the localization of triphosphoinositide and the inactivation of

leucocidin, and, if triphosphoinositide is involved in the interaction of leucocidin with the cell, the specificity of this must lie in the accessibility of the triphosphoinositide. Leucocidin induces a specific change in permeability to cations in the leucocyte. The possibility that this can follow a conformational change in triphosphoinositide is discussed by Woodin (1967).

The properties of the polyphosphoinositides are changed when they are added to leucocyte extracts. These appear to contain material that interferes with three different assay procedures and more than one substance may be involved. Their chemical nature is not known. The versatility of the chemical groupings in triphosphoinositide and the pronounced amphipathic properties of the intact molecule are such that complex-formation with other substances is to be expected. Triphosphoinositide is also present, in the cells we have studied, in very small amount and interference with polyphosphoinositide assay is not unexpected. It would seem that the assay of triphosphoinositide should always be controlled by an isotope-dilution technique.

Dawson (1965) has pointed out that, although isolated myelin is completely soluble in neutral chloroform-methanol, the extraction of triphosphoinositide from whole brain requires very acid conditions. Polyphosphoinositides are present in the neutral extracts of the leucocyte and erythrocyte membranes, and this may be because material capable of adsorbing polyphosphoinositides is removed in the cellular fractionation. However, until the absence of polyphosphoinositides from neutral extracts of other tissues has been confirmed, further comparison of our findings with the analysis of other tissues is not justified.

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REFERENCES

- Dawson, R. M. C. (1965). *Proc. 2nd Meet. Fed. Europ. biochem. Soc., Vienna*, vol. 2, p. 57.
- Dawson, R. M. C. & Eichberg, J. (1965). *Biochem. J.* **96**, 634.
- Dittmer, J. C. & Dawson, R. M. C. (1961). *Biochem. J.* **81**, 535.
- Eichberg, J. & Dawson, R. M. C. (1965). *Biochem. J.* **96**, 644.
- Hendrickson, H. S. & Ballou, C. E. (1964). *J. biol. Chem.* **239**, 1369.
- Hörhammer, L., Wagner, H. & Richter, G. (1959). *Biochem. Z.* **331**, 155.
- Prottey, C. & Hawthorne, J. W. (1966). *Biochem. J.* **101**, 191.

- Santiago-Calvo, E., Mulé, S., Redman, C. M., Hokin, M. R. & Hokin, L. E. (1964). *Biochim. biophys. Acta*, **84**, 550.
- Sastry, P. S. & Hokin, L. E. (1966). *J. biol. Chem.* **241**, 3354.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*, p. 149. Minneapolis: Burgess Publishing Co.
- Woodin, A. M. (1961). *Biochem. J.* **80**, 562.
- Woodin, A. M. (1966). *Exp. Cell Res.* **43**, 311.
- Woodin, A. M. (1967). In *The Biological Basis of Medicine*. Ed. by Bittar, E. London: Academic Press (Inc.) Ltd.
- Woodin, A. M. & Wieneke, A. A. (1966a). *Biochem. J.* **99**, 479.
- Woodin, A. M. & Wieneke, A. A. (1966b). *Biochem. J.* **99**, 493.
- Woodin, A. M. & Wieneke, A. A. (1967). *Biochem. J.* **105**, 1029.