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Modification of Erythrocyte Membranes by a Purified Phosphatidylinositol-Specific Phospholipase C (*Staphylococcus aureus*)

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A phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* was purified by a three-step procedure. The specific activity of the purified enzyme was approx. 6000 times that of the culture supernatant, with an overall recovery of approx. 10%. Estimation of the molecular weight by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and by gel filtration gave values of 33000 and 20000 respectively. A thiol group appears to be necessary for the activity of the enzyme. The purified enzyme had no detectable δ-haemolytic activity and was unable to hydrolyse *S. aureus* phospholipids. Phosphatidylinositol in erythrocyte 'ghosts' was readily hydrolysed by the purified phospholipase C. However, in contrast with our previous preliminary observations, phosphatidylinositol in intact erythrocytes was not significantly hydrolysed. These results suggest that at least 75–80% of the phosphatidylinositol is located at the inner leaflet of the membrane.

In a previous paper (Low & Finean, 1976) we reported on the potential of a phosphatidylinositol-specific phospholipase C present in *Staphylococcus aureus* culture supernatants as a tool for studying the distributions and functions in cell membranes of phosphatidylinositol, a phospholipid that has been suggested to be specifically involved in cell-surface receptor mechanisms (Michell, 1975).

We have now developed a purification procedure for this phospholipase and have repeated some of the preliminary observations on the effects of the enzyme on erythrocytes and erythrocyte 'ghosts'.

Experimental

Materials

Most materials were obtained from sources described previously (Low & Finean, 1976).

Lysozyme, pepsin, soya-bean trypsin inhibitor, haemoglobin, cytochrome *c* and bovine serum albumin were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Amberlite CG-50 (type II; 200 mesh) was obtained from BDH Ltd., Poole, Dorset, U.K. Before use (or re-use) the resin was washed with 2×25 vol. of water, 10 vol. of 0.1 M-NaOH, 2×25 vol. of water and finally 2×10 vol. of equilibrating buffer (0.15 M-NaCl/0.05 M-sodium acetate/acetic acid, pH 5.5). In the buffer washes the pH was maintained at 5.5 with acetic acid. Sephadex G-75 (superfine grade) was obtained from Pharmacia (G.B.) Ltd., London W.5, U.K.

Lipid extracts of *S. aureus* were prepared from bacteria harvested during the production of culture supernatants (see under 'Methods') as described previously (Low & Finean, 1976).

Methods

Methods, except where specifically mentioned, were as described previously (Low & Finean, 1976).

Preparation of S. aureus culture supernatant. A peptone diffusate was prepared by dissolving 88 g of Difco proteose peptone in 200 ml of water and dialysing against 2 litres of water for 24 h. The diffusate, together with ammonium lactate (20 g), KH_2PO_4 (4 g), K_2HPO_4 (4 g) and $MgSO_4,7H_2O$ (1.6 g), was made up to a final volume of 4 litres with water and the pH adjusted to 7.4 with NaOH. The medium was distributed between four 2-litre flasks and autoclaved. Each flask was inoculated with 10 ml of an overnight culture of *Staphylococcus aureus* (Newman) and incubated on an orbital shaker (250 rev./min) for 24 h at 37°C. The bacteria were removed by centrifugation.

Chromatography on Amberlite CG-50. All subsequent operations were carried out at 4°C. NaCl (20 g) was added to the culture supernatant and the pH adjusted to 5.5 with acetic acid. The culture supernatant was then passed through a column (approx. 38 cm×2.6 cm) of Amberlite CG-50 equilibrated in 0.15 M-NaCl/0.05 M-sodium acetate/acetic acid buffer, pH 5.5 (see under 'Materials'), at a flow rate of approx. 150 ml/h. The column was washed with 2 litres of equilibrating buffer and then eluted with a linear gradient formed from 400 ml of equilibrating buffer and 400 ml of 1 M-trisodium citrate at a flow rate of 30 ml/h. Phospholipase C was eluted as a broad peak (approx. 150–350 ml eluted) and fractions containing more than 20 units/ml were pooled for further purification.

Chromatography on Sephadex G-75. The pooled fractions were concentrated with an Amicon ultrafiltration cell (model 52), by using a PM-10 mem-

brane, to a volume of approx. 2ml. The concentrate was then centrifuged (15000*g* for 30min) to remove insoluble material and the supernatant applied to a column (89cm×2.6cm) of Sephadex G-75 (superfine grade) equilibrated in 50mM-Tris/HCl, pH 7.4. The column was eluted at a flow rate of 8ml/h and 4ml fractions were collected. The A_{220} and phospholipase C activity of the fractions are shown in Fig. 1. Fractions containing more than 20units/ml were pooled and stored at −20°C.

Assay for phospholipase C activity. Phospholipase C activity was determined by measuring organic phosphorus release as described previously (Low & Finean, 1976). Incubation mixtures contained 0.5ml of sonicated soya-bean lipid (20mg/ml), 0.2ml of 100mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)-ethanesulphonic acid]/NaOH, pH 7.0, and 0.3ml of enzyme in a final volume of 1ml. Culture supernatants were dialysed overnight at 4°C against 1000vol. of water (to remove phosphate buffer) before being assayed. Fractions from columns were assayed directly without prior dialysis. A unit was defined as the amount of activity releasing 0.1μmol of organic phosphorus into the upper phase in 2h at 37°C (Low & Finean, 1976).

Phospholipase C activity in polyacrylamide gels was measured by a similar procedure. Gels were soaked for 2h in water to remove gel buffer and then sliced into 5mm sections. The sections were crushed with a glass rod and mixed with substrate, buffer and water (0.2ml) as above. Incubation was for 20h at 37°C and the organic phosphorus released was determined as in the standard assay.

Phospholipase C activity towards *S. aureus* phospholipids was measured by substituting a sonicated suspension of the extracted lipids (20mM-lipid phosphorus) for soya-bean 'phosphatidylcholine' in the standard assay (Low & Finean, 1976).

Treatment of erythrocyte membranes with phospholipase C. Incubation conditions were essentially as described by Low & Finean (1976). The phospholipase C used in all these experiments was obtained from the Sephadex G-75 column (see above), 0.3ml of pooled fractions being used in a 1ml incubation. Control incubations contained 0.3ml of 50mM-Tris/HCl, pH 7.4. In the incubations with erythrocytes, additional sucrose was added to maintain iso-osmoticity. Incubation was at 37°C and the reaction was stopped by cooling on ice for 2min, followed by the rapid addition of 3.75ml of chloroform/methanol (1:2, v/v). After 30min extraction at room temperature, the extract was split into two phases by the addition of 1.25ml each of chloroform and 2M-KCl. The whole of the lower phase was taken for analysis by chromatography as before (Low & Finean, 1976). In each experiment erythrocytes and erythrocyte 'ghosts' were prepared from the same sample of blood (Low & Finean, 1976).

In our previous paper (Low & Finean, 1976) lipid extracts of erythrocytes were prepared by adding methanol (2.5ml) followed by chloroform (1.25ml). This modified extraction procedure avoided the formation of a rubbery mass of haemoglobin, which was obtained when the chloroform and methanol were added together. However, it was subsequently found that this procedure allowed rapid hydrolysis by the phospholipase C (approx. 50% in 30s) of the membrane phosphatidylinositol, in the interval between the addition of the methanol and the chloroform. This rapid hydrolysis did not take place when chloroform and methanol were added together. This experimental artifact probably accounts for our previous observation that a proportion of the phosphatidylinositol in intact erythrocytes was accessible to the phospholipase C (Low & Finean, 1976). In the present experiments, therefore, all extractions were made by the standard procedure (see above).

Polyacrylamide-gel electrophoresis. Electrophoresis in 7% polyacrylamide gels was performed as described by Reisfeld *et al.* (1962). Gels were either stained for protein or assayed for phospholipase C activity (see above).

Electrophoresis in 11% polyacrylamide gels in the presence of sodium dodecyl sulphate was performed as described by Weber & Osborn (1969). For mol.wt. estimations bovine serum albumin (67000), pepsin (35000), soya-bean trypsin inhibitor (21500), haemoglobin (17000) and lysozyme (14000) were used as standards. Gels were stained for protein with 0.25% Coomassie Brilliant Blue.

Assay for haemolytic activity. δ-Haemolytic activity was assayed essentially as described by Caird & Wiseman (1970), by incubating 1ml of purified phospholipase C (in 50mM-Tris/HCl, pH7.4, adjusted to iso-osmoticity with 3M-NaCl) with 1ml of a 2% (v/v) suspension of human erythrocytes (washed and suspended in 0.15M-NaCl/10mM-H_3PO_4/NaOH, pH7.3). One haemolytic unit produces 50% haemolysis during incubation for 2h at 37°C, followed by 16h at 4°C. Because of the low protein concentration in the purified phospholipase C preparations, the maximum amount of protein that could be included in the assays was 10μg.

Assay for protein. Protein was generally assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The protein concentration in fractions obtained from the Sephadex G-75 column were too low to be assayed by this method, and A_{220} measurements were used instead, with bovine serum albumin as standard.

Chromatography of lipids. Neutral lipids of *S. aureus* were analysed by t.l.c. on silicic acid in the solvent light petroleum (b.p. 40–60°C)/diethyl ether/acetic acid (80:20:1, by vol.).

Table 1. *Purification of phosphatidylinositol-specific phospholipase C from S. aureus*
For experimental details see under 'Methods'. Culture supernatants were dialysed before phospholipase C activity was assayed. Dialysed culture supernatants contain approx. one-quarter the amount of protein shown in the Table.

	Volume (ml)	Protein (mg)	$10^{-3}\times$Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification
Culture supernatant	3900	16400	18.0	1.07	100	1
Amberlite CG-50	210	147	13.0	89	72	83
Concentration	1.8	6.1	3.2	525	18	490
Sephadex G-75	24	0.24	1.6	6660	9	6170

Results

Purification of phospholipase C

Table 1 summarizes the purification achieved by the procedure described in detail in the Experimental section.

The first step, with Amberlite CG-50, is particularly effective, as it enables relatively large volumes of culture supernatant to be concentrated and purified in a single step. Other methods of concentrating the culture supernatant [i.e. precipitation with ethanol or $(NH_4)_2SO_4$] were tried, but gave poor purification. The optimum amount of culture supernatant that can be loaded on the column is about 4 litres. If larger amounts of culture supernatant are used with a column of this size a considerable proportion of the phospholipase C does not bind and the subsequent purification is also relatively poor.

Substantial loss of activity occurs during concentration of the pooled Amberlite CG-50 fractions by ultrafiltration. Although a substantial amount of low-molecular-weight peptide material passed through the membrane this did not contain appreciable phospholipase C activity. Further, there was no significant phospholipase C activity in the precipitate that formed during concentration. Attempts to improve the recovery in this concentration step by carrying out the ultrafiltration at pH 7.4 or by freeze-drying the pooled fractions were unsuccessful. $(NH_4)_2SO_4$ precipitation of the pooled fractions gave slightly better recovery, but the concentrate had a lower specific activity and after chromatography on the Sephadex column gave a less pure product.

The poor overall recovery of phospholipase C activity may reflect the instability of the purified enzyme: incubation of the purified phospholipase C at 37°C for 30min results in a 75% loss of activity. Similarly, storage at −20°C for 6 weeks causes approx. 40% of the phospholipase C activity to be lost.

Purity and properties of phospholipase C

The elution of phospholipase C from the Sephadex column in the same region as a peak of A_{220} (Fig. 1) suggested that the phospholipase C was the major protein component of the purified preparation. This was confirmed when purified phospholipase C was analysed by polyacrylamide-gel electrophoresis. The mobility of the phospholipase C was similar to that of the major protein band (Fig. 2), and from the relative intensities of the bands we estimate the enzyme to be approx. 90% pure.

The mobility of this major protein band in polyacrylamide gels containing sodium dodecyl sulphate indicated a molecular weight of approx. 33000. However, the behaviour of the phospholipase C on the Sephadex column (calibrated with cytochrome *c*, soya-bean trypsin inhibitor and pepsin) indicated a mol.wt. of approx. 20000.

The phospholipase C may have a thiol group which is necessary for activity, as preincubation with $HgCl_2$, $CuSO_4$ or *p*-chloromercuribenzoate results in a considerable loss of activity (Table 2). However, more extensive studies of this type are complicated by the sensitivity of the enzyme to preincubation itself (see above).

δ-Haemolytic activity could not be detected in the purified phospholipase C (the maximum amount that could be included in the assay was 10μg). This low activity contrasts sharply with that obtained (approx. 12000 haemolytic units/mg) with the purified δ-haemolysin preparation reported to contain phosphatidylinositol-specific phospholipase C (Caird & Wiseman, 1970), although other workers have generally reported δ-haemolysin specific activities in the range 100–400 haemolytic units/mg (for references see Wiseman, 1975).

The purified phospholipase C did not hydrolyse phospholipids extracted from *S. aureus*. Incubation of the extracted lipids (10μmol of lipid phosphorus/ml) with 20 units of the purified enzyme/ml for 2h at 37°C released less than 10nmol of water-soluble organic phosphorus, indicating that, under these conditions, less than 0.1% of the phospholipids was hydrolysed. The possibility that only lipid products might be formed (e.g. by hydrolysis of diphosphatidylglycerol to phosphatidylglycerol phosphate and diacylglycerol) appears unlikely, as t.l.c. showed no significant increase in the amount of diacylglycerol during the treatment with phospho-

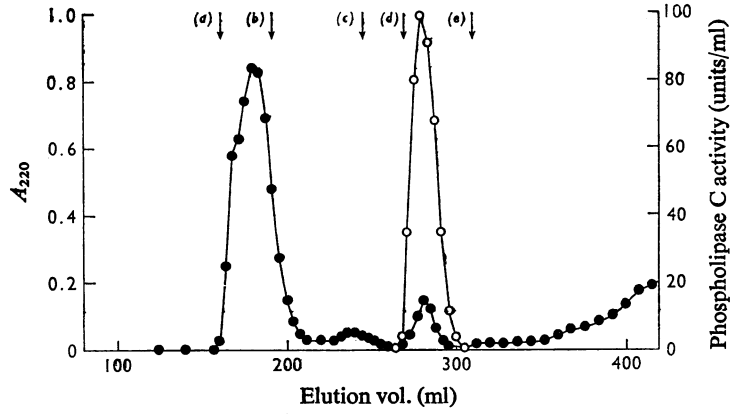

Fig. 1. *Chromatography of phospholipase C on Sephadex G-75*

Phospholipase C from the Amberlite CG-50 column was concentrated to approx. 2ml (for details see the text) and applied to a column (89cm×2.6cm) of Sephadex G-75 (superfine grade) equilibrated in 50mM-Tris/HCl, pH 7.4, and eluted at 8ml/h. ●, A_{220}; ○, phospholipase C activity. The elution positions of (*a*) Blue Dextran, (*b*) pepsin, (*c*) cytochrome *c* (dimer), (*d*) soya-bean trypsin inhibitor and (*e*) cytochrome *c* are indicated by arrows on top of the elution diagram.

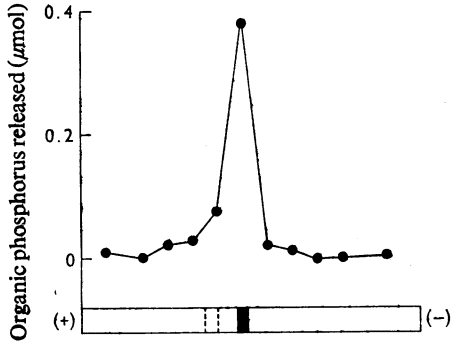

Fig. 2. *Location of phospholipase C activity after polyacrylamide-gel electrophoresis*

Purified phospholipase C (70 units) was electrophoresed as described by Reisfeld *et al.* (1962). Non-stained gels were sectioned and assayed for phospholipase C activity (see under 'Methods'). The values shown are the means of determinations carried out on duplicate gels. The positions of the components visible at this loading (in stained gels) are shown in the drawing.

Table 2. *Effect of thiol inhibitors on the activity of purified phospholipase C*

Purified phospholipase C (2.5 units) was preincubated with various inhibitors for 5min at 37°C in the standard phospholipase C assay mixture (minus substrate). Concentrations refer to those present during the preincubation. Control preincubations ('No additions') show approx. 30% loss of activity. Values are the means of duplicate determinations in two separate experiments (*a* and *b*). —, Not tested.

Additions	Concn. (mM)	Relative phospholipase C activity (%) (*a*)	(*b*)
No additions	—	100	100
$HgCl_2$	0.04	90	—
	0.2	4	22
$CuSO_4$	0.2	36	55
	2.0	12	—
Lead acetate	2.0	65	—
p-Chloromercuribenzoate	0.2	—	100
	2.0	9	0

lipase C (see above). The latter method would have detected hydrolysis of approx. 1% of the phospholipid phosphodiester bonds.

Action of purified phospholipase C on erythrocyte membranes

The ability of the purified phospholipase C to hydrolyse phosphatidylinositol in erythrocyte 'ghosts' is shown in Fig. 3. With sheep (and possibly pig) erythrocyte 'ghosts' there appears to be a resistant fraction (approx. 15%), whereas essentially all (i.e. >95%) of the ox erythrocyte 'ghost' phosphatidylinositol is hydrolysed. A more detailed analysis of this difference is prevented by the difficulty of measuring small amounts of phosphatidylinositol [PI/PL ratio (phosphatidylinositol/other phospholipids) less than 0.5%] with any degree of accuracy.

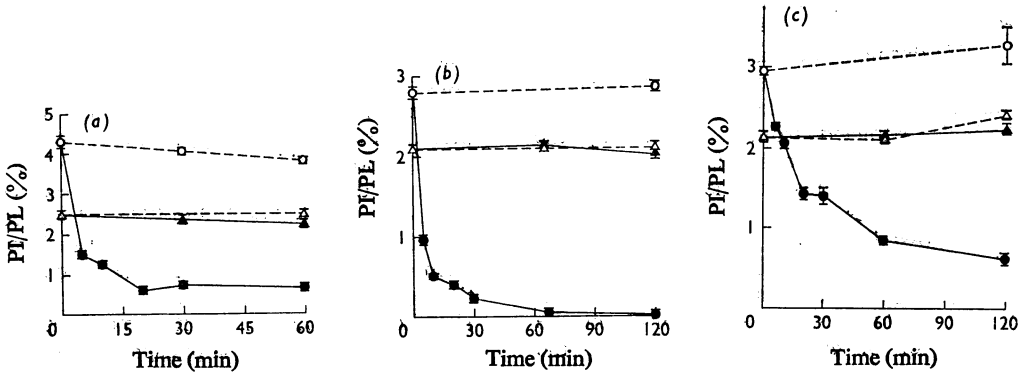

Fig. 3. *Effect of purified phospholipase C on the phosphatidylinositol content of erythrocyte membranes*
(*a*) Sheep erythrocyte membranes; (*b*) ox erythrocyte membranes; (*c*) pig erythrocyte membranes; ○, ●, erythrocyte ghosts; △, ▲, erythrocytes; ○, △, control incubations; ●, ▲, incubations with phospholipase C [(*a*) 21 units/ml; (*b*), (*c*) 12 units/ml]. PI/PL is the amount of phosphatidylinositol (PI) expressed as a percentage of the other phospholipids (PL). Values are the means of triplicate incubations ± S.E.M.

The phosphatidylinositol in the intact erythrocytes, however, showed little or no hydrolysis during the same incubation period. The small decrease in phosphatidylinositol content (approx. 10%) observed with sheep erythrocytes (Fig. 3*a*) may not be significant. Another experiment with a different batch of cells showed less than 5% decrease. Haemolysis, in all these experiments, was less than 2%.

The phosphatidylinositol/total phospholipid ratio of erythrocyte 'ghosts' is generally significantly higher (25–50%) than that of the erythrocytes from which they were prepared (compare control incubations in Fig. 3). Smaller increases in this ratio also occur sometimes when control erythrocytes are simply incubated. These increases are probably due to the dephosphorylation of polyphosphoinositides, which occurs when erythrocytes are haemolysed (Buckley & Hawthorne, 1972; Garrett *et al.*, 1975).

Discussion

The purification procedure for the phosphatidylinositol-specific phospholipase C from *S. aureus* described in the present paper permits the preparation of enzyme of sufficient purity and activity for membrane-modification studies. These same procedures can undoubtedly be refined to improve the purity further, but for this it will probably be necessary to study also the factors required for the stabilization of enzyme activity.

The molecular weight of the phospholipase C has been estimated by both sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (approx. 33000) and gel filtration (approx. 20000). However, at present we have no indication as to which of the factors (charge, shape, carbohydrate constituents, etc.) known to affect estimation of molecular weights by these methods is responsible for this discrepancy.

The low haemolytic activity of the purified phospholipase C emphasizes that it is unlikely to be related to the δ-haemolysin studied by Caird & Wiseman (1970) and reported to hydrolyse phosphatidylinositol. In addition, our phospholipase C has a much lower molecular weight (see above) than that estimated for the δ-haemolysin (approx. 200000). The phospholipase activity reported for the δ-haemolysin must be attributed either to contamination with our phosphatidylinositol-specific enzyme or to the existence of another phospholipase C which is inherently haemolytic and can be identified with the δ-haemolysin, but, in the absence of information about the specific activity of their phospholipase (Caird & Wiseman, 1970), it is not possible to decide which explanation is correct. It should be noted, however, that phospholipase activity was not detected in δ-haemolysin purified by other workers (Heatley, 1971; Kreger *et al.*, 1971; Kapral, 1972; Rahal, 1972).

There is as yet no clear indication of what the normal function of this phosphatidylinositol-specific phospholipase C might be. It cannot be assumed, on the basis of our failure (Low & Finean, 1976) to detect hydrolysis of erythrocyte membrane phospholipids other than phosphatidylinositol, that this phospholipid is the natural substrate for the enzyme. *S. aureus* has not been reported to contain any phosphatidylinositol (for references see Beining *et al.*, 1975), but it is still possible that the phospholipase C that we have purified could be involved in the catabolism of other *S. aureus* phospholipids. The

observation (Kusaka, 1975) that a substantial proportion of *S. aureus* phospholipid was hydrolysed to diglyceride during protoplast formation certainly seems to suggest the involvement of a phospholipase C. However, our failure to detect the hydrolysis of phospholipids extracted from *S. aureus* by the purified phosphatidylinositol-specific phospholipase C would suggest that this enzyme is not the one involved in this process.

A further possibility is that the enzyme has toxic properties as a direct consequence of its potential ability to hydrolyse mammalian cell phosphatidylinositol and could therefore be involved in the pathogenicity of *S. aureus*. In this respect the observation (Doery *et al.*, 1965) that the phosphatidylinositol-specific phospholipase C was produced by coagulase-positive haemolytic (i.e. 'pathogenic') strains of *S. aureus* and not by coagulase-negative non-haemolytic strains may be relevant.

Our objective in isolating and purifying this phosphatidylinositol-specific phospholipase C has been to obtain an enzyme suitable for studying the distribution and functions of this phospholipid in cell membranes, and in our previous paper (Low & Finean, 1976) we reported some preliminary experiments in which as much as 60% of erythrocyte phosphatidylinositol appeared to have been hydrolysed in the intact erythrocyte without significant haemolysis. However, it is now apparent that hydrolysis of phosphatidylinositol by the phospholipase C continued during the early stages of the lipid-extraction procedure (see under 'Methods') and thus invalidated the earlier observations. A more thorough investigation has now shown that, although the enzyme will hydrolyse 85–95% of the phosphatidylinositol in erythrocyte 'ghosts', there is no significant hydrolysis when it is applied to the intact cell.

Increased accessibility of molecular components of erythrocyte membranes to chemical labels and hydrolytic enzymes during formation of 'ghosts' has generally been taken to indicate a location at the internal surface of the membrane (Zwaal *et al.*, 1973; Zwaal & Roelofsen, 1976). A similar interpretation can be applied to the present results, where it is clear that more than 90% of the erythrocyte phosphatidylinositol but only 15% (less than 5% in the ox) of the 'ghost' phosphatidylinositol is inaccessible to the phospholipase C. If it is assumed that phosphatidylinositol that is inaccessible in the 'ghost' is also inaccessible in the intact erythrocyte, it can be calculated that at least 75–80% (approx. 95% in the ox) of the erythrocyte phosphatidylinositol is inaccessible to the enzyme and possibly located at the inner surface of the membrane. The distribution of the phosphatidylinositol that is not hydrolysed in either the intact erythrocyte or the 'ghost' (15% in the sheep) cannot, of course, be deduced from the present results. It is likely, however, that the additional phosphatidylinositol that appears to be produced during formation of the 'ghosts' is derived from di- and tri-phosphoinositide, which would be expected to be located at the inner surface of the membrane, where ATP is available for phosphorylation (Schneider & Kirschner, 1970; Buckley & Hawthorne, 1972). A direct demonstration of the internal location of these phosphorylation reactions has been reported by Garrett & Redman (1975).

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