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# A STRUCTURAL STUDY OF THE MODIFICATION OF ERYTHROCYTE GHOSTS BY PHOSPHOLIPASE C

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SUMMARY

Treatment of rat and human erythrocyte ghosts with phospholipase C (EC 3.1.4.3) hydrolysed individual phospholipids at different rates. The more ionic lipids, phosphatidyl serine and phosphatidyl inositol, were little affected.

Dense droplets associated with the membranes were observed by phase contrast microscopy and by electron microscopy and they have been identified with the diglyceride and ceramide formed by the hydrolysis of phospholipids. A significant shrinkage of the ghosts was also noted.

The modifications of lipid components have been used to identify lipid and lipoprotein bands in the X-ray diffraction patterns of dried preparations. Implications regarding the molecular organisation of the membrane and the mode of action of phospholipase C on membranes have been considered.

## INTRODUCTION

Phospholipase C (EC 3.1.4.3) from *Clostridium welchii* has been used to modify a variety of membrane preparations including erythrocytes[1–6], liver[7] and ascites tumour[4] plasma membranes, microsomal preparations from liver[8], muscle[5,9], stomach[10] and intestine [11] and liver mitochondria[6].

These modifications have been shown to suppress the activity of $(Na^+-K^+)$-ATPase[1,2,4,7,10,11] but have little or no effect on $Mg^{2+}$-dependent ATPase in these systems. The activities of $Mg^{2+}$-dependent ATPase and $Ca^{2+}$ accumulation are suppressed in sarcoplasmic reticulum microsomes[9,12] and that of glucose-6-phosphatase in liver microsomes[8]. In most of these cases the hydrolysis of phospholipids was extensive and in certain cases the enzyme studied was shown to be inactivated rather than destroyed since the addition of lipid to the treated preparation caused whole or partial reactivation[8,9,12]. In the experiments with muscle microsomes 60–70 % of the total phospholipid, including over 90 % of the membrane phosphatidyl choline, was hydrolysed[13] and electron micrographs provided evidence of a separation of the diglyceride so produced as small droplets which remained associated with the membranes[14].

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Phospholipase C-modified membranes have also been studied by optical rotatory dispersion and circular dichroism techniques. LENARD AND SINGER[2] failed to detect any changes in circular dichroism spectra and concluded that the protein configuration was unaffected. GORDON *et al.*[4], however, demonstrated slight changes both in circular dichroism and optical rotatory dispersion spectra. Both groups of authors assumed that the diglyceride and ceramide produced by the hydrolysis remained associated with membrane protein and suggested that these experiments emphasised a dominance of non-polar interaction between lipid and protein in the membrane.

However, electron micrographs of phospholipase C-treated muscle microsomes have demonstrated the possibility that the diglyceride would form separated lipid droplets that were no longer part of the membrane structure, although still physically associated with the membranes[14], and a similar effect was readily demonstrated in the case of haemoglobin-free human erythrocyte ghosts[5,6] and of mitochondria[6].

Our own observations have now been extended to provide a more quantitative assessment of the hydrolysis of specific membrane phospholipids by phospholipase C in rat and human erythrocyte membranes prepared by lysis at different osmolarities[15]. Since it has proved possible to obtain X-ray diffraction data from condensed samples of erythrocyte membranes[16] this technique was used in addition to electron microscopy in order to assess the accompanying changes in structural organization.

## MATERIALS AND METHODS

### *Preparation of membranes*

Two types (Types I and II) of erythrocyte membranes were prepared from both rat and human erythrocytes[15]. The rat blood was freshly drawn into heparin and human erythrocytes (usually less than 4 days and never more than 3 weeks old and all O+) were obtained from a local blood bank.

Type I human erythrocyte membranes were obtained by haemolysis in 2 imosM bicarbonate buffer (pH 6.15) while Type I rat erythrocyte membranes were obtained by haemolysis in 2 imosM bicarbonate buffer (pH 6.8). Type II membranes from both rat and human erythrocytes were obtained using 10 imosM bicarbonate or Tris–HCl buffers (pH 7.4). In addition, ghosts especially suitable for quantitative assessment of dimensional changes were prepared using 80 imosM bicarbonate buffer. All membranes were well washed with the buffer used for lysis.

### *Treatment of membranes with phospholipase C*

Phospholipase C (*Cl. welchii* Type I, Sigma Chemical Co.) was dissolved at the required concentration in 6.66 mM $CaCl_2$ (pH 7.0). 1 vol. of this was added to 10 vol. of a membrane suspension containing 1 mg protein/ml and incubated at 37° for 30 min. For most experiments phospholipase C concentrations from 0.1 to 30 $\mu$g enzyme per mg membrane protein were employed. Control incubations omitted only the phospholipase C. The digestion was terminated by cooling the membrane suspension to 0°, and sedimenting at 100000 $\times$ $g_{av.}$ for 30 min. The membrane-free supernatant was removed for assay of phosphate and of protein. The membranes were washed in the appropriate haemolysing buffer and resedimented at 100000 $\times$ $g_{av.}$ for 30 min.

Samples to be used for lipid extraction and for electron microscopy were taken directly from this sediment, while samples for X-ray diffraction analysis were resuspended in the appropriate haemolysing buffer and sedimented at 150000 $\times$ $g_{av.}$ to provide a more compact pellet.

*Phosphorus determination*

Since in the erythrocyte membranes there is little phosphorus-containing material other than phospholipid, the extent of hydrolysis of phospholipid was estimated from phosphorus analyses[17] of untreated membranes relative to that of membrane-free supernatants after phospholipase digestion.

*Protein determination*

Protein was determined by the method of LOWRY *et al.*[18] or by the biuret method of WEICHSELBAUM[19].

*Lipid extraction and chromatography*

Total lipid extracts were prepared according to the methods of RAND AND LUZZATI[20] and GARBUS *et al.*[21] (omitting phosphate from the wash solution). For some purposes the total lipid extract was separated into phospholipid and neutral lipid by dissolving in 4 % methanol in chloroform (v/v) and applying to a silicic acid column (Silicar cc-7 Mallinkrodt 100–200 mesh) with a column loading $>$ 1 $\mu$g phosphorus/g silicic acid. The column was washed with 10 column volumes of 4 % methanol in chloroform to obtain a fraction which contained mainly neutral lipids. Phospholipids were then eluted from the column with methanol.

For assessment of phospholipids the extracts were examined by thin-layer chromatography[22]. The amount of each phospholipid component (visualised with iodine) was estimated by scraping the spots, digesting with $HClO_4$ and determining the amount of phosphate present in each spot[23]. Neutral lipids were also identified by thin-layer chromatography[24].

Lipid samples for X-ray diffraction studies were prepared by taking the appropriate lipid extracts to dryness under vacuum at 30–40°. Lipid was then scraped from the side of the flask with a spatula and mounted across the gap in a suitable specimen holder[16].

*Phase contrast microscopy*

Samples of control and modified membranes were examined with a Zeiss photomicroscope. The membrane sample was diluted in the appropriate buffer and the fully hydrated membranes were routinely examined at a magnification of 1250 $\times$. The photomicrographs were recorded at a magnification of 400 $\times$.

*Electron microscopy*

Material for sectioning was fixed with glutaraldehyde (6.25 %, pH 7.5) followed by $OsO_4$ (1 % in cacodylate buffer (pH 7.5), 3 h). It was dehydrated in a graded series of alcohols followed by propylene oxide and embedded in Araldite. Sections were stained with methanolic uranyl acetate (25 %) prior to examination in the electron microscope.

Material for immediate observation was fixed by adding a drop of $OsO_4$ to a drop of membrane suspension deposited on a carbon-covered specimen grid. After

2 min the drop was largely removed and replaced immediately by a drop of distilled water. This drop was replaced several times before allowing the sample to dry completely. The fixed membranes were then examined directly in the electron microscope.

*X-ray diffraction*

Samples for X-ray diffraction studies were mounted on brass sample holders and condensed by partial dehydration in a controlled humidity chamber[16] located in the wide-angle or low-angle cameras[25]. Patterns were recorded at intermediate (Type I only) and fully dried stages.

## RESULTS

*Hydrolysis of membrane components*

The effects of phospholipase C treatment in terms of amount of phosphate released during a 30-min incubation with increasing concentrations of phospholipase C were very similar for all types of membrane studied (Fig. 1).

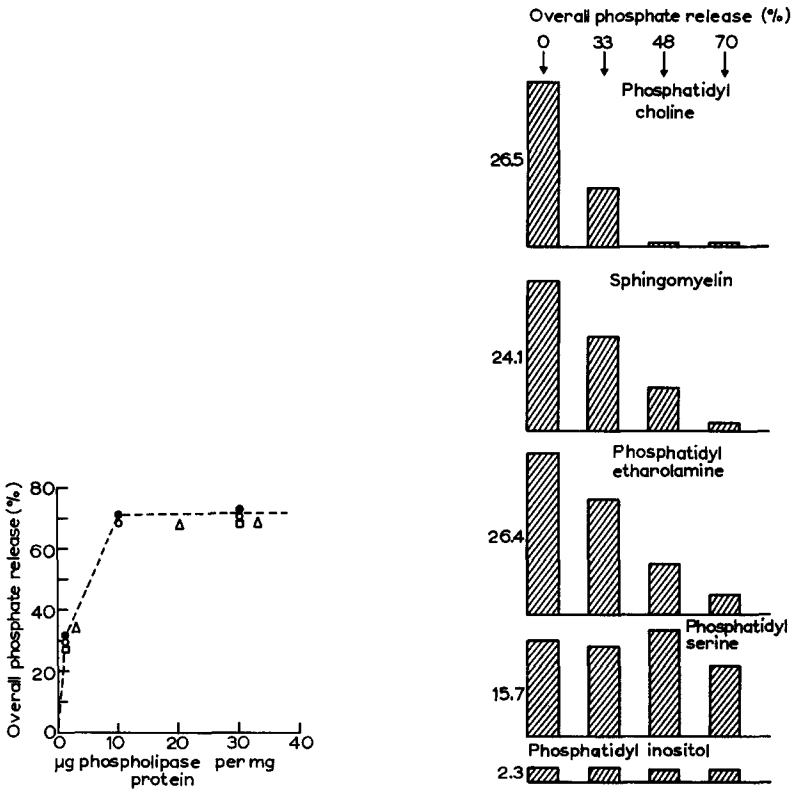


Fig. 1. Effect of phospholipase C concentration upon release of organic phosphate. Treatment as in MATERIALS AND METHODS. Composite diagram includes data from: ●, rat Type I; ○, rat Type II; □, human Type I; △, human Type II erythrocyte membrane preparations.

Fig. 2. Quantitative estimation of extent of hydrolysis of individual membrane phospholipids at various levels of treatment with phospholipase C. Figures above columns indicate % of total phosphate released at each level of treatment. Figures on left of columns represent the proportion of each phospholipid in the original membrane. The heights of the columns are scaled in relation to these proportions. Human Type II erythrocyte ghost preparation. Mean of two experiments.

Thin-layer chromatographs of lipid extracts corresponding to various levels of phosphate release indicated clearly that phosphatidyl choline was hydrolysed most rapidly (Fig. 2). The apparent susceptibility to hydrolysis in all cases appeared to be in the order, phosphatidyl choline > sphingomyelin > phosphatidyl ethanolamine. The more ionic lipids, phosphatidyl serine and phosphatidyl inositol did not show appreciable degradation, even at very high levels of phospholipase treatment. The sphingomyelin of human erythrocyte ghosts appeared to be hydrolysed more readily than that of the rat erythrocyte ghosts.

Thin-layer chromatography for neutral lipids demonstrated that diglycerides and ceramide were formed during phospholipase digestion.

Analyses of membrane-free supernatants showed no release of protein or peptides during phospholipase C digestion.

*Phase contrast microscopy and electron microscopy*

Examples of phase contrast micrographs of control and modified erythrocyte ghosts are shown in Figs. 3 and 4. Fig. 5 is an electron micrograph of phospholipase C-treated erythrocyte ghosts which have been lightly fixed with $OsO_4$ before drying down on the electron microscope grid.

Dense droplets 3000–10000 Å in diameter were seen to be associated with the phospholipase C-treated membranes. It was also apparent that extensive treatment with phospholipase C had caused an appreciable shrinkage of the ghosts. This shrinkage was analysed quantitatively in the case of ghosts prepared in 80 imosM bicarbonate buffer. The control ghosts were spherical and of fairly constant dimensions (Fig. 6). Analysis of the dimensions of the phospholipase C-treated (maximum) ghosts showed a marked shift to smaller diameters (Fig. 6). The corresponding reduction in surface area was calculated to be at least 45 %. Measurements made on electron micrographs gave very similar values for the shrinkage.

Electron micrographs of sectioned material also showed dense droplets associated with phospholipase C-treated membranes (Figs. 7 and 8), and at high magnifications many of the dense droplets could be seen to have an ordered fine structure.

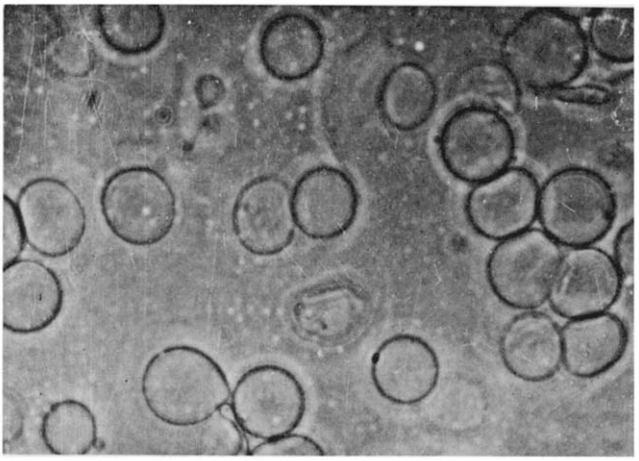

Fig. 3. Phase contrast micrograph of untreated sample of erythrocyte membranes. Human Type I preparations. Final magnification 1450 ×.

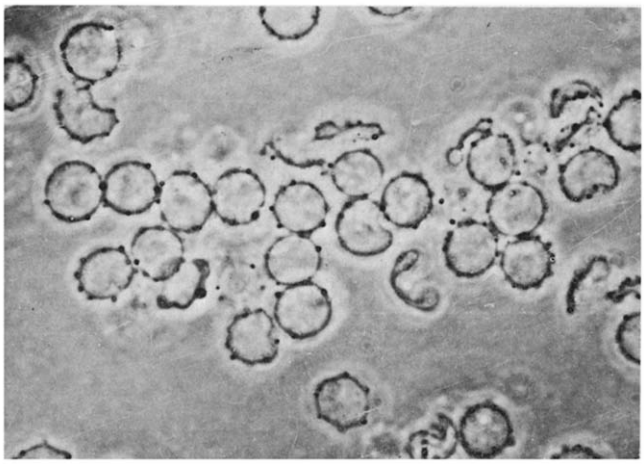

Fig. 4. Phase contrast micrograph of phospholipase C-treated erythrocyte membranes. Human Type I preparation. Final magnification 1450 ×.

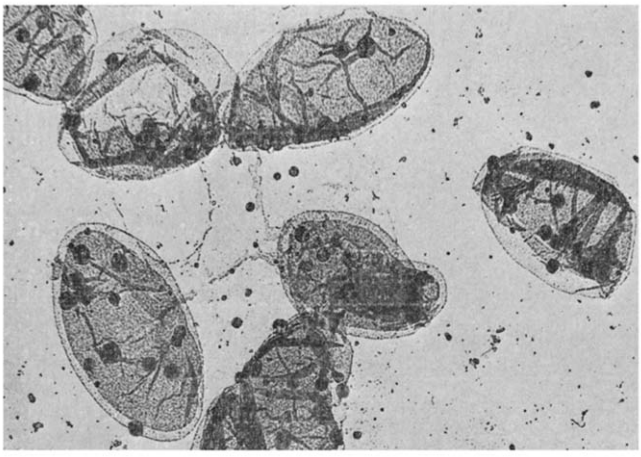

Fig. 5. Electron micrograph of phospholipase C-treated erythrocyte membranes. Human Type II preparation. Ghosts were fixed with $OsO_4$ and deposited on the specimen grid (see MATERIALS AND METHODS). Final magnification approx. 4500 ×.

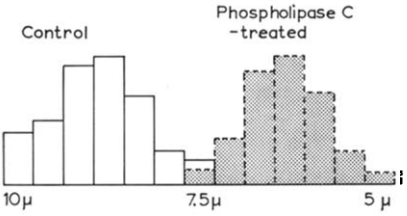


Fig. 6. Examples of histograms showing distributions of diameters in control and phospholipase C-treated samples. Erythrocyte ghosts prepared in 80 imosM bicarbonate buffer. Measurements were made on phase contrast photomicrographs of randomly selected areas of each sample at a magnification of about 1000 ×. 50–100 ghosts were measured in each sample and all experiments showed this clear separation of size distributions.

This structure was better-defined in the case of treated rat ghosts than in the case of treated human ghosts. Many droplets showed a regular layered structure with a periodicity of about 60 Å and in some there was an approximately hexagonal distribution of dense spots at about 70 Å centre-to-centre separation (Fig. 8).

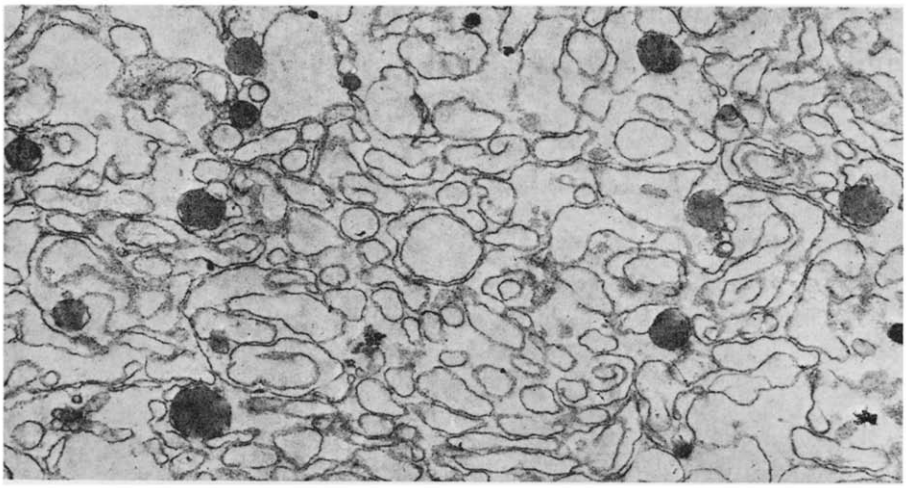

Fig. 7. Electron micrographs of phospholipase C-treated erythrocyte membranes. Human Type II preparation. $OsO_4$-fixed material. Thin sections stained with uranyl acetate. Final magnification 10 000 ×.

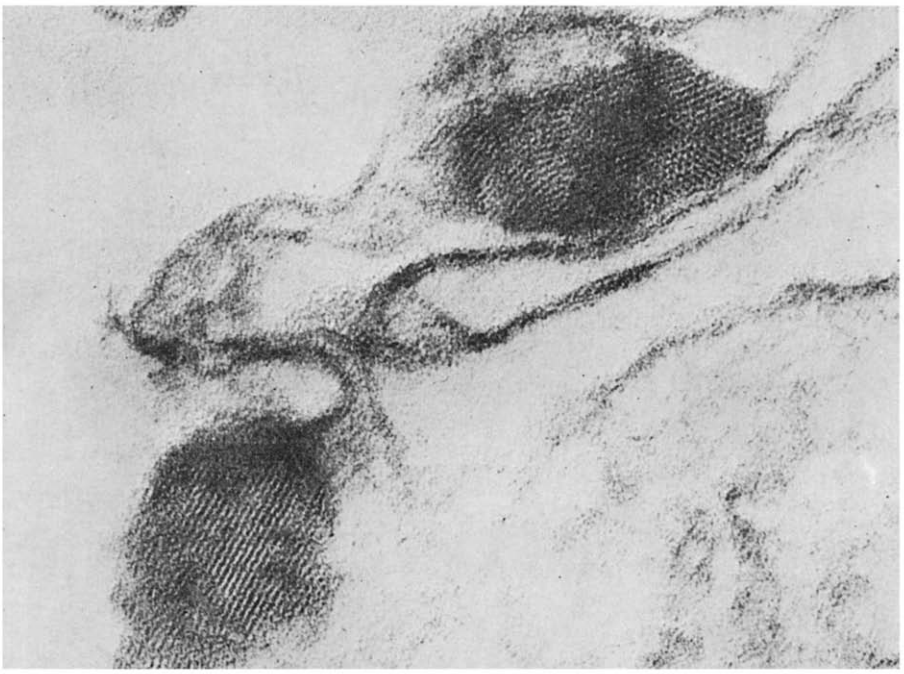

Fig. 8. Electron micrographs of phospholipase C-treated erythrocyte membranes. Rat Type II preparation. $OsO_4$-fixed material. Thin sections stained with uranyl acetate. Final magnification 160 000 ×.

The trilamellar characteristic of the membrane itself remained well-defined following phospholipase C treatment and the dimensions were not significantly changed.

*X-ray diffraction*

In the low-angle diffraction studies of Type I rat and human erythrocyte ghosts which had been treated with high levels of phospholipase C a lamellar-type diffraction pattern was recorded at hydration levels greater than about 30 % with respect to final dried weight. The periodicity was only a little lower than that of the control preparation (105 Å as compared with 110 Å).

Dried samples of phospholipase C-treated Type I and Type II preparations of rat and human erythrocyte ghosts which had lost less than 10 % of the membrane phosphorus gave X-ray reflexions at 80–85, 53 and 43 Å, and frequently, after long standing, also a very sharp reflexion at 34.5 Å. This is essentially the normal pattern obtained from control preparations (Fig. 9a). Low-angle patterns from rat ghosts which had lost 20 % membrane phosphorus showed reflexions at 65 and 49 Å instead of at 53 and 43 Å whilst samples which had been hydrolysed to the extent that more than 25 % of the membrane phosphorus had been released showed reflexions at 80–85, 49 and 34.5 Å (Fig. 9c). The latter reflexion was much more intense than it was in the control preparations and was always evident in the pattern of the freshly dried sample. Comparable preparations of human ghosts showed well-defined reflexions only at 80–85 and 34.5 Å (ref. 5). Diffraction in the 40–60 Å region was diffuse and weak.

The wide-angle X-ray diffraction patterns from the phospholipase C-treated membrane preparations also showed some striking changes. The control samples gave only broad reflexions at about 4.6 and 10–11 Å even down to the freshly dried

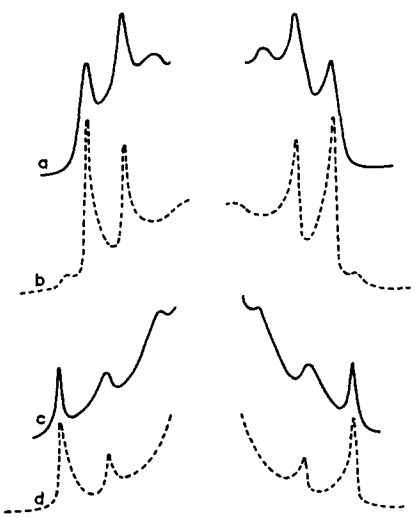


Fig. 9. Microdensitometer traces of low-angle X-ray diffraction patterns. Rat Type II preparation. a, untreated membranes, dried state; b, total lipid extract of a, dried state; c, phospholipase C-treated membranes, dried state; d, neutral lipid fraction of total lipid extract of c, dried state.

state (some better-defined reflexions were occasionally observed after long standing). Phospholipase C-treated samples which had lost more than 20 % of the membrane phosphorus gave additional sharp reflexions which became evident even before drying was completed. The detail of the changes in wide-angle patterns of the modified membranes and of their lipid extracts was very complex and is reported and interpreted here only in general terms.

The dried samples of lipid extracts from control and phospholipase C-treated membranes gave low-angle and wide-angle X-ray reflexions which showed significant correspondence with those from the membrane samples. The total lipid extract from the control membranes gave low-angle reflexions at 53 and 43 Å (Fig. 9b) whilst the lipid from maximally treated rat membranes gave instead a reflexion at 49 Å. The total lipid extracted from maximally treated human membranes, when examined at room temperature, gave only ill-defined diffraction in the 40–60 Å region. The most significant correspondence between the low-angle diffraction patterns of modified membranes and lipid extracts came from a comparison of the low-angle patterns of the dried preparations of phospholipase-treated rat erythrocyte membranes and the neutral lipid from these membranes (Figs. 9c and 9d). Reflexions at 50 and 34.5 Å were prominent in both patterns, but the dried membranes also gave an additional reflexion at 80–85 Å.

## DISCUSSION

The action of phospholipase C on phospholipids of erythrocyte membranes as observed in these experiments is in general agreement with respect to specificities and extent of hydrolysis with other studies on comparable erythrocyte membranes[2,4,6,30] and on isolated lipid samples[26,27].

The main products of hydrolysis are phosphoryl choline and phosphoryl ethanolamine and neutral lipids (diglyceride and ceramide). The observations made by phase contrast microscopy of the freshly treated ghosts and by electron microscopy of $OsO_4$-fixed material strongly suggest that the neutral lipid forms droplets 3000–10000 Å in diameter which remain associated with the membranes. That the droplets observed by microscopy are indeed neutral lipid, is supported by the isolation[5] from the sonicated membrane preparation of a fraction enriched in diglyceride and also showing an increased concentration of dense droplets. Similar droplets of somewhat smaller diameters have previously been observed in association with phospholipase C-treated muscle microsomal membranes and again a diglyceride-enriched fraction was isolated[14]. Other workers[2,4] have also observed that the neutral lipid sediments with the treated erythrocyte membranes but seem to have assumed that the neutral lipid remained associated with other membrane components in a way that was of significance in relation to the nature of the lipid–protein interaction in the intact membrane. The present experiments indicate that the neutral lipid formed by phospholipase C digestion of membrane phospholipid immediately accumulates in droplets which, although still physically associated with residual membranes, are most unlikely to be involved in any significant way in the molecular organisation of the membrane. Any observed changes in molecular organisation of the membrane must therefore be considered in relation to the removal of the whole

of the phospholipid molecule from the membrane structure and not just to the removal of the ionic end group.

The ordered fine structure of many of the membrane-associated droplets is in keeping with the suggestion that they are lipid droplets. The fine structure resembles that observed in phospholipid systems that contain very small amounts of water[28,29] and also in droplets (probably ceramide trihexoside) observed in cases of Fabry's disease (V. W. STEWARD, personal communication).

The fact that the fine structure is more clearly defined in droplets associated with treated rat membranes than it is in those from human membranes probably reflects a difference in the chemical nature of the neutral lipids derived from the two sources. Such a difference is also indicated by the low-angle X-ray diffraction patterns of the dried preparations of modified membranes. In the case of the rat preparation the neutral lipid is probably represented by the intense and well-defined reflexion at 49 Å whilst in the pattern of the human preparation one can detect only very faint and ill-defined reflexions in this region. Gas chromatographic studies of lipid extracted from erythrocyte membranes[30] have shown that there is appreciably less polyunsaturation (in particular C18:2) among the lipids from rat material as compared with human and such a factor would be expected to lead to a lower solidification temperature so that at room temperature rat neutral lipids would be expected to be more ordered than the human material. This could account both for the more detailed fine structure seen in electron micrographs and for the more clearly defined X-ray reflexions.

The present studies also provide some points of direct significance in relation to the molecular organisation of the membranes. For example, the structure of the membrane must be such as to allow the phospholipase C to interact specifically with the ionic end group of the phospholipid molecules. Moreover, the observation that all of the components of the phospholipid molecule become displaced from the membrane during enzymic degradation could be taken to suggest that the non-polar portions of the phospholipid molecule may also need to become associated with the enzyme. This would be in keeping with the fact that phospholipase C does not hydrolyse glyceryl phosphoryl choline[31]. In general, the observations would seem to favour a molecular model in which the phospholipid molecules are at least partially available at the membrane surface. They would also favour models in which the ionic end groups which provide the main specificity of the substrate molecules are directed outwards towards the membrane surface.

More specific suggestions concerning the arrangement of lipid and protein components in the membrane come from quantitative considerations of the decrease in area of the erythrocyte ghost which results from the hydrolysis of the phospholipid. A substantial contraction of area of the membrane takes place without any appreciable increase in the thickness as observed both by electron microscopy and by X-ray diffraction. There are no major changes of protein chain configuration detectable by optical methods[2,4] and no release of protein from the membrane. Such observations would seem to suggest that any protein layer (or any other non-lipid component) in the structure must have an open configuration that is capable of lateral contraction without an increase in thickness. Such an open structure might allow access of the enzyme molecule to an underlying layer containing phospholipid molecules.

It is also of interest to note that in the low-angle X-ray diffraction pattern of the dried membrane preparations the so called 'residual lipoprotein band' persists following phospholipase C digestion. It appears to be the "labile" phospholipid that is most affected. In a previous paper[5] we have tentatively suggested that this could mean that the labile lipid can be identified as being predominantly choline phospholipids and to a less extent phosphatidyl ethanolamine and that the residual lipoprotein involves the more acidic lipids which are little affected by phospholipase C digestion. These more polar lipids might be specifically associated with protein in regions where protein overlies part of the lipid layer whilst the less polar lipid molecules may be exposed in spaces in the protein matrix and retained in the membrane through non-polar lipid–lipid interactions and interactions with non-polar segments of the protein which may penetrate the lipid layer at the perimeters of these exposed regions.

Some indication of the proportion of the total area of the membrane occupied by lipid components can be obtained by comparing the observed overall shrinkage of the membrane with the proportion of lipid removed at maximum hydrolysis (70 %) of the phospholipid. If treatment with phospholipase C should displace not only phospholipid but also cholesterol and other lipids in the same proportions then the area occupied by the lipid in the membrane would be reduced by about 70 %. The overall reduction in the area of membrane observed in these experiments was 45 % which would indicate that lipid occupied at least 65 % of the membrane area. If any of the lipid components are displaced from the membrane in proportions lower than the extent of phospholipid hydrolysis then this figure of 65 % would be increased. In the event of only phospholipid being displaced from the membrane by the action of phospholipase C the figure could rise to 100 %, and lipid would be considered to form an uninterrupted layer throughout the membrane.

In similar experiments with muscle microsomes[14] a 60–70 % hydrolysis of phospholipid was found to cause a maximum decrease of membrane area of 55 %. In this case the lipid of the membrane is almost entirely phospholipid and lipid must therefore occupy at least 80 % of the total area of the membrane.

Further experiments of this kind should establish more precisely the proportions of the areas of membranes occupied by lipid components but the figures available already clearly indicate that the bulk of the protein of some membranes must overlie the lipid layer. Some recent molecular models accommodate protein in the same plane as the lipid[2,3,32,33]: the present observations would indicate that this can occur only to a minor extent in the membranes so far studied.

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