PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Structural Studies Relating to the Distribution of Molecular Components in Erythrocyte Membranes

J. B. Finean, R. Freeman and A. R. Limbrick

Phil. Trans. R. Soc. Lond. B 1974 **268**, 15-21
doi: 10.1098/rstb.1974.0012

Phil. Trans. R. Soc. Lond. B. **268**, 15–21 (1974) [15]
Printed in Great Britain

Structural studies relating to the distribution of molecular components in erythrocyte membranes

By J. B. Finean, R. Freeman and A. R. Limbrick
Department of Biochemistry, University of Birmingham

[Plates 6–7]

Hydrolysis of phospholipid in erythrocyte membranes by phospholipase C (*Cl. perfringens* or *B. cereus*) leads to the formation of membrane-attached droplets which contain at least some of the lipid products of hydrolysis and possibly other membrane lipids which can no longer be retained in the phospholipid-depleted membranes. A similar effect is observed when isolated membrane lipids are treated with phospholipase C and appears to represent a phase separation resulting from the change in lipid composition. Droplets and residual membranes have been isolated from phospholipase C-modified preparations for chemical analysis.

The structural parameters of the residual membranes as observed by electron microscopy and by low angle X-ray diffraction appear to be little changed. This suggests that the residual lipid is able to condense to maintain molecular continuity within the membrane and that the protein components are also able to condense laterally so as not to change the membrane thickness, although they probably do contribute to a change in electron-density distribution which is detected in the X-ray diffraction studies.

These observations are further discussed in relation to recent reports on molecular parameters of specific membrane components and suggestions concerning molecular distributions within the membrane.

Information on the distribution of molecular components in membranes is gradually accumulating from a variety of studies of the *in situ* labelling or degradation of both lipid and protein components. This information is most extensive in the case of the erythrocyte membrane.

It is already widely accepted that the lipid in the erythrocyte membrane is in bilayer form and that while some protein components are largely superficial to the lipid layer others penetrate deep into or even through the hydrocarbon region of the bilayer. Suggestions relating to the localization of individual molecular components in relation to this general structural picture have come largely from comparisons of their availability either for chemical labelling or for specific enzymic degradation by agents which can have access to one or to both sides of the membrane. The reliability of the conclusion is dependent on the specificities of interactions of the agents and knowledge of the permeability of the cell membrane preparation to such agents.

Displacement of membrane lipid by phospholipase action

Observations of the action on the erythrocyte membrane of phospholipases, and especially of those phospholipases (phospholipase C and sphingomyelinase) which remove the phosphate-containing end group to leave a lipid residue with greatly reduced polar characteristics, have provided some information on the distribution of phospholipid molecules. The action of phospholipase C preparations from *Clostridium perfringens* or from *Bacillus cereus* has been observed by phase-contrast microscopy and electron microscopy (Coleman, Finean, Knutton & Limbrick 1970) to lead to the formation of optically dense droplets which remain attached to the surface of the membrane (figure 1, plate 6). These droplets were suggested to contain at least some of the lipid degradative products which had been displaced from the molecular organization of the membrane. On the other hand, when erythrocyte membranes were treated with purified sphingomyelinase from *Staphylococcus aureus* droplet formation was not observed (Colley,

Zwaal, Roelofson & van Deenen 1973) and the ceramide resulting from the hydrolysis of sphingomyelin was presumed to have stayed within the membrane structure. In the experiments with phospholipase C, droplet formation was observed to be accompanied by a decrease in ghost diameters (Coleman *et al.* 1970) and a tentative calculation of the area occupied in the membrane by lipid was made on the assumption that the reduction in membrane area was related directly to a displacement of lipid in proportion to the extent of phospholipid hydrolysis. More precise information on the composition of the displaced lipid droplets and on the content of the residual membranes will soon be available from analyses of fractions obtained from phospholipase C-modified human erythrocyte ghosts following disruption by extensive sonication. Preliminary analyses of the droplet fraction (figure 2, plate 6) have indicated that the droplets contain some cholesterol as well as diglyceride but there appears to be little ceramide present.

Previous studies of the associations of phospholipid and cholesterol (Bourges, Small & Dervichian 1967; Ladbrooke, Williams & Chapman 1968) had indicated that cholesterol would be unlikely to maintain a single phase with phospholipid at molecular ratios greater than about 1:1. Consequently when a high proportion of the phospholipid in erythrocyte membranes was hydrolysed to diglyceride which was then displaced from the membrane structure it might have been anticipated that cholesterol too would be displaced either to form a mixed phase with the diglyceride or, if the solubility of cholesterol in diglyceride should be low, to form a separate cholesterol phase. However, other groups of workers (Horwitz, Krut & Kaminsky 1971; Green & Green 1973) have since demonstrated that ratios of at least 2:1 cholesterol: phospholipid can be maintained in dilute dispersions of cholesterol and phospholipid in water and our own unpublished X-ray diffraction studies of phospholipid:cholesterol, phospholipid:diglyceride and diglyceride–cholesterol systems have demonstrated that the highest cholesterol:phospholipid molecular proportions that can be maintained as a single diffracting phase are influenced by the choice of solvents in which the components are mixed before drying down or dispersing in water. Cholesterol:phospholipid molecular ratios of up to 2:1 can be achieved in systems which appear by X-ray diffraction to involve only a single phase. Nevertheless, when lipids isolated from erythrocyte membranes are dispersed in water and treated with phospholipase C uniformly dense droplets (figure 3, plate 6) quickly appear in association with the liposomes or myelinics and the residual lipid layer contracts. The changes closely parallel those seen with the phospholipase C-treated membranes and emphasize that the phase separation is an inevitable physical consequence of the hydrolysis of a substantial proportion of the phospholipid to diglyceride.

In the case of ghosts prepared from freshly collected rat and pig erythrocytes there is an added complication in that these membranes carry an active diglyceride lipase which converts a substantial proportion of the diglyceride produced by phospholipase C action to monoglyceride and fatty acid (Michell, Coleman & Finean 1973). Droplets visible by phase-contrast microscopy still form at the surface of the phospholipase C-treated membranes and it is assumed that they probably contain monoglyceride and fatty acid as well as diglyceride, cholesterol and possibly ceramide. Such droplets have proved to be much more difficult to separate from the modified rat and pig membranes than from the human ones. Electron micrographs of these modified membrane preparations have frequently demonstrated droplets or granules which have a regular internal structure (figure 4, plate 6), but neither the condition which leads to the formation of these structures nor their composition have yet been established.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

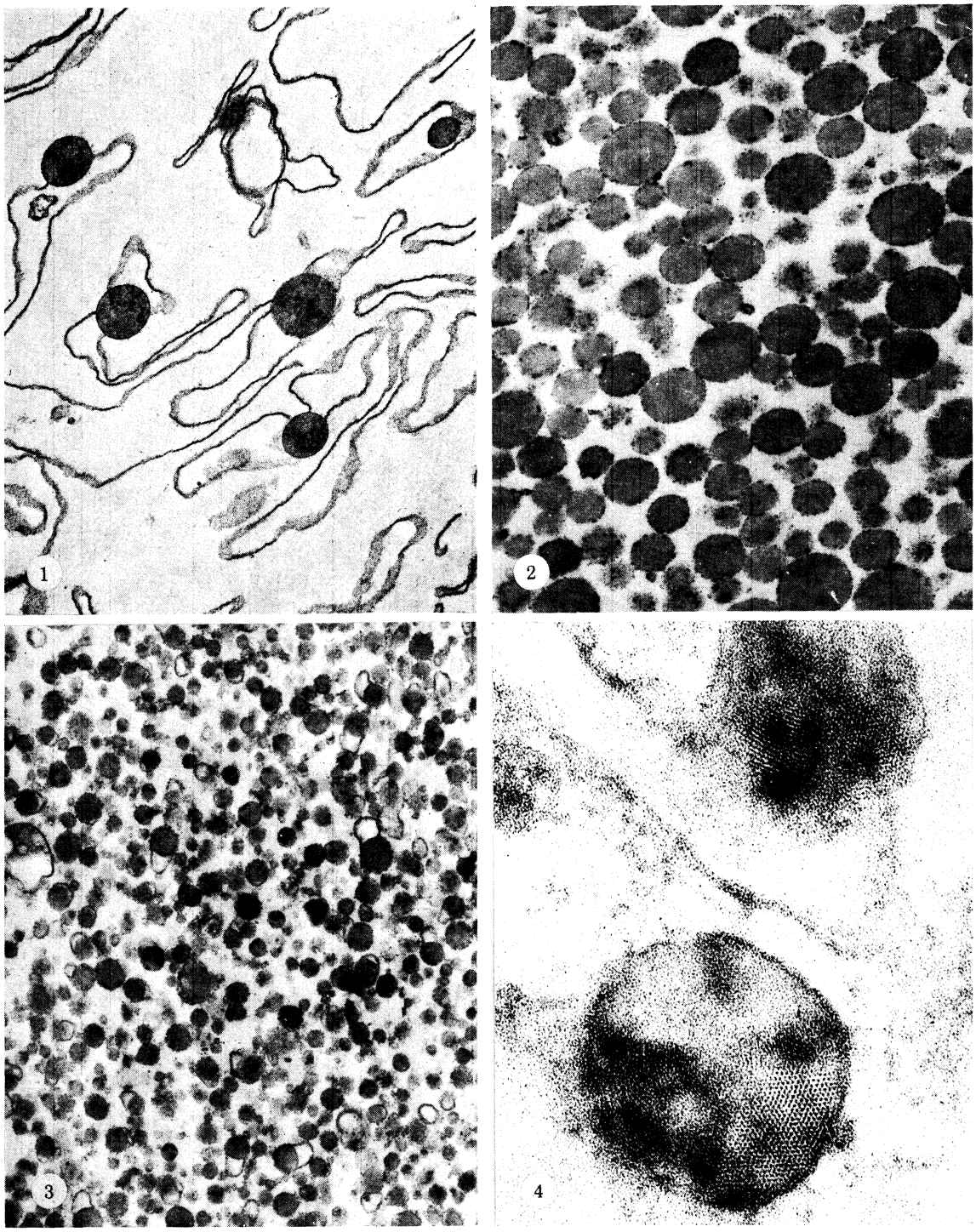


FIGURE 1. Electron micrograph of a section through a haemoglobin-free preparation of human erythrocyte ghosts treated with phospholipase C (*Cl. perfringens*) to maximum phospholipid hydrolysis (~ 70%) (magn. × 30 000).

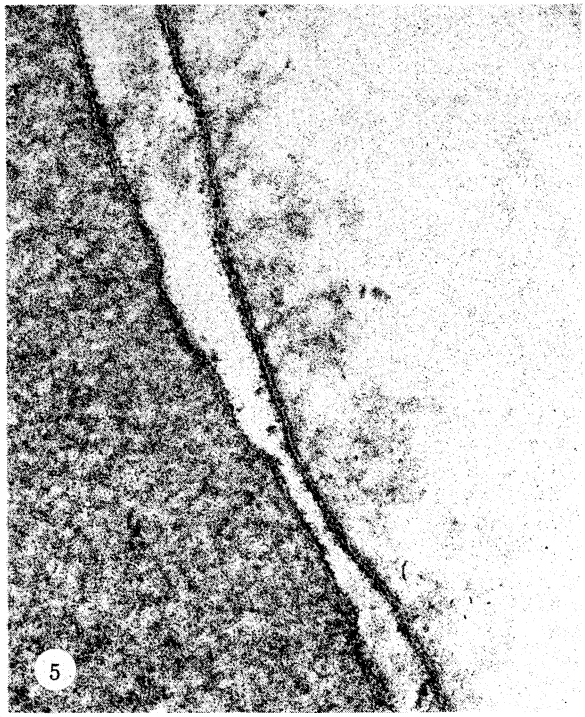
FIGURE 2. Electron micrograph of a section through a droplet fraction separated from a sonicated preparation of phospholipase C (*Cl. perfringens*) – treated human erythrocyte ghosts (magn. × 30 000).

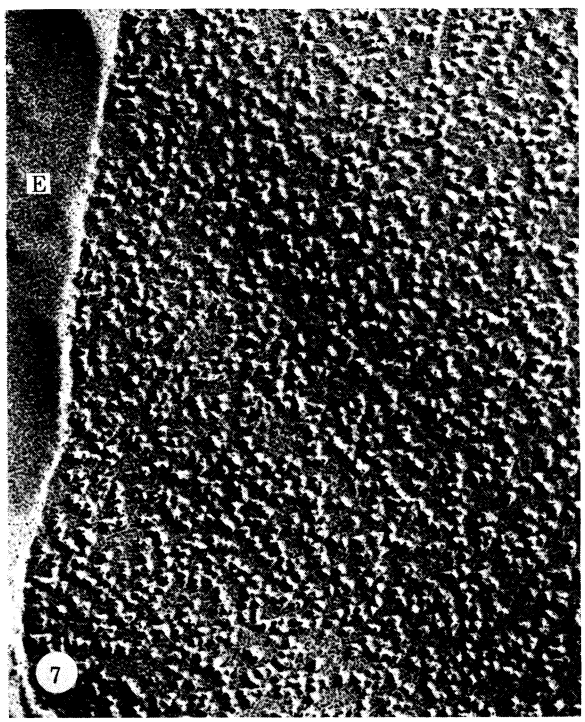
FIGURE 3. Electron micrograph of a section through a phospholipase C (*Cl. perfringens*)-treated aqueous dispersion of lipid extracted from human erythrocyte ghosts (magn. × 30 000).

FIGURE 4. Electron micrograph of a section through structured droplets in a phospholipase C (*Cl. perfringens*)-treated preparation of pig erythrocyte ghosts (magn. × 160 000).

(*Facing p.* 16)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES





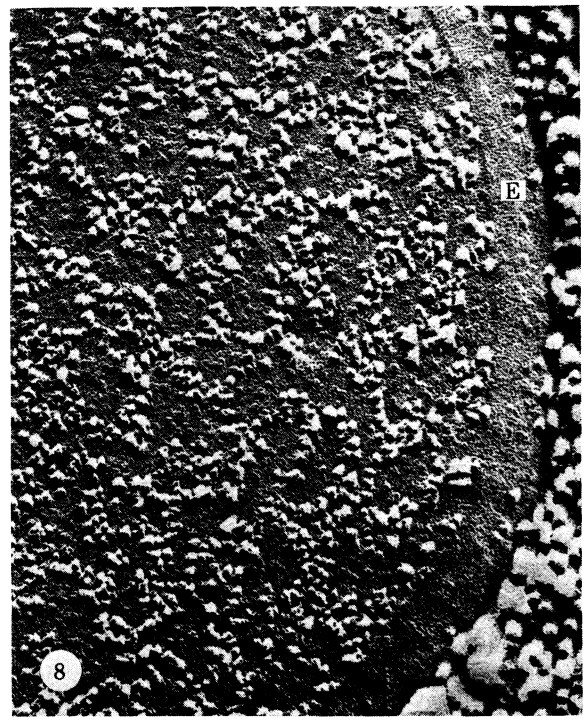


FIGURE 5. Electron micrograph of a section through a sample of human erythrocyte ghosts prepared in 80 mosmol/l buffer and left at room temperature for a few minutes to allow a proportion of the ghosts to reseal before treatment with phospholipase C (*B. cereus*). The ghost on the left had resealed and appeared not to have been modified by the phospholipase C. The ghost on the right carried droplets such as those illustrated in figure 1 which are indicative of phospholipid hydrolysis. (Magn. × 130 000.)

FIGURE 6. A selected area showing close-packed membranes in an electron micrograph of a section through a preparation of pig erythrocyte ghosts which had been haemolysed in less than 3 mosmol buffer and centrifuged at 500 000 *g* for 100 min (Magn. × 110 000).

FIGURES 7 and 8. Electron micrographs of freeze-etched samples of human erythrocyte ghosts. In each case the direction of shadowing is from below. E indicates the etched outer face of the membrane. The main area represents the A fracture face. Figure 7, control preparation; figure 8, preparation modified by mild phospholipase C (*Cl. perfringens*) treatment.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Structural characteristics of lipid-depleted membranes

In electron micrographs of sections of control and modified erythrocyte ghosts the trilamellar feature of membrane structure is more clearly defined following phospholipase C treatment but the overall dimension is not significantly different (figure 5, plate 7). This would suggest that the membrane structure is such as to be able to accommodate the loss of a substantial proportion of the lipid content and still maintain the principal structural parameters.

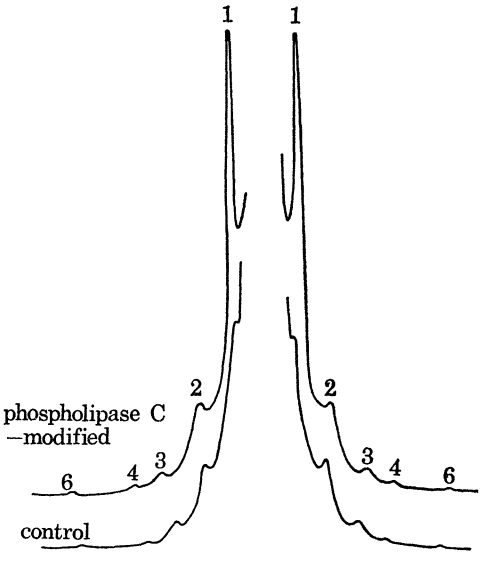


FIGURE 9. Microdensitometer trace across the well-defined low-angle reflexions in the X-ray diffraction patterns of control and phospholipase C (*Cl. perfringens*)-modified preparations of pig erythrocyte membranes. Membranes prepared in buffer strength of less than 3 mosmol/l.

This suggestion is also supported by some X-ray diffraction data obtained from very compact pellets (70–80 % water) of erythrocyte membranes obtained by high speed (70 000 rev/min) centrifugation of erythrocyte ghosts prepared at very low osmolarities. Such ghosts retain a considerable amount of haemoglobin and, although they appear intact and spherical in the dispersed state, when centrifuged they condense to form regions of concentric, close-packed membranes (figure 6, plate 7) which yield well-defined X-ray diffraction patterns.

The membrane system is essentially the same as that previously obtained (Finean, Coleman, Green & Limbrick 1966; Knutton, Finean, Coleman & Limbrick 1970) when low osmolarity ghosts (type I) were allowed to condense by slow dehydration down to about 30 to 50 % hydration and the close-packed layering seen in electron micrographs of thin sections of the two types of preparation appear identical. Such layering as seen in electron micrographs consists of trilamellar units resembling closely those seen in the dispersed ghosts but brought together with dense lines in close apposition to form a symmetrical repeating layer (figure 6, pl ate 7).

Ghosts prepared at higher osmolarities (above 5 mosmol/l) do not exhibit this spontaneous condensation to form a compact layering and the X-ray diffraction patterns, obtained only after partial dehydration, are much less well defined (Knutton *et al.* 1970).

The tightly packed layering gives a series of well-defined low-angle diffraction bands (figure 9) which can be accounted for as orders of a single periodicity. For the control preparation this periodicity is about 11.7 nm and electron micrographs show a symmetrical membrane unit

about 10 nm thick. Samples treated with phospholipase C also centrifuge to give a very compact membrane pellet which again features regions of closely packed membranes and yields well-defined diffraction patterns. The sharp low-angle reflexions (figure 9) relate to a periodicity of 11 nm and differ from the control preparation mainly in an intensification of the first-order reflexion. The modified sample also gives a series of broader reflexions (1.3 nm, 0.97 nm) at slightly higher angles and new sharp reflexions (0.59 nm, 0.39 nm) in the high-angle region. These sharp wide-angle rings may relate to a cholesterol phase.

A simple comparison of diffraction patterns from control and phospholipase C-modified preparations indicates that the membrane thickness has been little changed by the treatment but there has been a change in electron-density distribution which emphasizes the first order diffraction of the lamellar periodicity.

The fact that the membrane decreases substantially in area but maintains its original thickness when lipid is displaced from it by the action of phospholipase C probably indicates that the residual lipid components condense to maintain structural continuity within the membrane and that the protein is also able to condense laterally without increasing the overall thickness. For protein lying outside the lipid bilayer boundaries this would imply an increased density which may well be responsible for the relative increase in the first-order diffraction band.

Attempts have been made to observe the predicted lateral condensation of protein (or glycoprotein) components directly by applying the freeze-fracturing technique for electron microscopy (Limbrick & Knutton 1971). One problem encountered in this study was that when the phospholipid of erythrocyte ghosts had been maximally hydrolysed by treatment with phospholipase C (*Cl. perfringens*) the membranes failed to fracture along their internal hydrophobic interfaces and internal fracture faces could not be exposed. Small areas of fracture face could be observed at lower levels of hydrolysis and these showed a marked aggregation of the normally uniformly dispersed granular projections (figures 7, 8, plate 7): it was not possible to make a quantitative overall assessment of the extent of condensation that this limited view of aggregation represented. The fact that fracturing along the interior of the membrane is inhibited by phospholipase C treatment does, however, support the idea that the change in molecular arrangement involves the creation of a higher concentration of non-lipid molecules at the interior of the membrane. The lateral condensation of such protein or glycoprotein components should also be demonstrable through the application of appropriate surface labels which can be seen in the electron microscope.

Erythrocyte membrane structure

The implications of the phospholipase C modification studies in relation to the general distribution of lipid and protein or glycoprotein components in the erythrocyte membranes have previously (Finean 1972) been expressed in terms of an adaptation (figure 10) of a model published by Zahler (1969). Some more specific identification of protein molecules might now be attempted in the light of recent successes in the *in situ* labelling and degradation of protein components and identification through polyacrylamide gel electrophoresis. In particular, two polypeptide chains have been shown to be available for labelling on both sides of the membrane and must therefore span the bilayer (Bretscher 1971; Segrest, Kahane, Jackson & Marchesi 1973). One of these components has been identified with the principal glycoprotein component of the membrane and detailed information is now available on the distribution of amino acid residues in the polypeptide chain. Regions of predominance of polar or of non-polar

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

regions have been identified and this is such as to suggest that a central, predominantly non-polar region of the chain might serve to span the lipid bilayer so that the two terminal polar regions might coil on the surface to form an unequal dumb-bell shaped molecule as illustrated in figure 11. Such molecules might be expected to float independently in the lipid phase and to show a condensation effect when part of the lipid is removed. Detailed molecular characterizations of this kind are required to refine the molecular picture of the membrane.

Some attempts have also been made to establish the location of different types of lipids in the membrane more precisely. The above model allows the distinguishing of two lipid situations, one in direct association with protein or glycoprotein components and one free of such direct

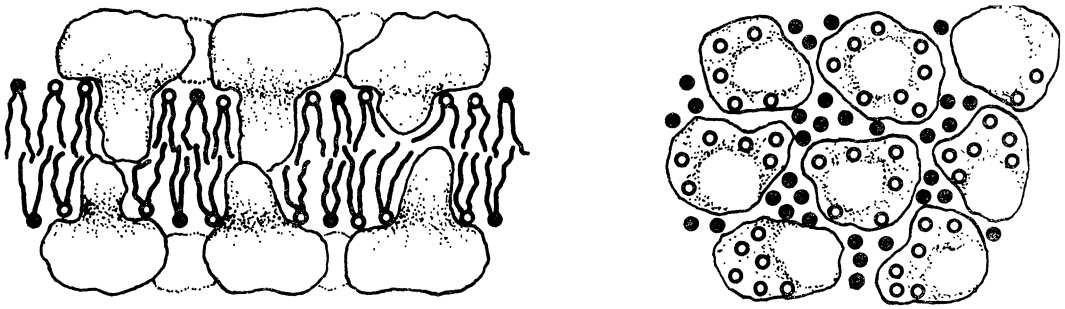


FIGURE 10. General molecular model of an erythrocyte membrane (Finean 1972). Mushroom- or dumb-bell shaped protein molecules penetrate into or through a lipid bilayer. The head-groups of lipids are distinguished as open or filled circles according to whether or not they are in a position to interact with the protein.

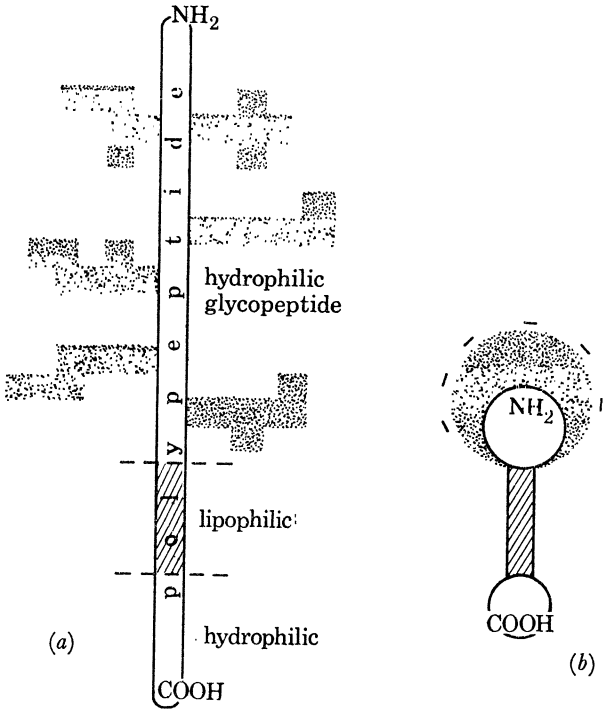


FIGURE 11. Impressions of characteristics of glycoprotein molecules described by Segrest *et al.* (1973). (*a*) Molecule fully extended to distinguish characteristics of individual sections. (*b*) Molecule folded so as to maintain a cylindrical segment of predominantly hydrophobic polypeptide chain to span the lipid bilayer and to form compact globular structures from the more polar terminal segments. Relative dimensions shown are realistic for a side view of the folded molecule.

associations. Where there is evidence of a requirement of a specific type of lipid for an activity involving also a protein component such as an enzyme protein the position of at least a part of this lipid component can, in a limited sense, be defined, but such data are largely qualitative and not very extensive. It has been suggested that the lipid in this model that is not directly associated with protein might represent the lipid that is most readily exchangeable or most readily degradable by lipases. It could therefore include a high proportion of the cholesterol and the less polar phospholipids of the membrane. However, in the absence of a detailed understanding of the phenomena referred to, these suggestions must be regarded as largely speculative. A more substantially based suggestion that sphingomyelin and phosphatidyl choline are the dominant lipids in the outer half of the lipid bilayer and the more ionic lipids located mainly in the inner half has been made partly on the evidence of limited specific lipid labelling experiments and partly on some characteristics of the action of phospholipases (Bretscher 1972*a*, *b*). However, the labelling experiments could equally well be explained in terms of a more ready access to lipid components in general from the inner surface of the membrane and recent studies of conditions required for the action of a variety of purified phospholipases on intact erythrocytes and erythrocyte ghosts (Sabban, Laster & Loyter 1972; Woodward & Zwaal 1972; Colley *et al.* 1973; Low, Limbrick & Finean 1973) lead us to suggest that the location of lipids in the membrane is unlikely to be the sole or even the dominant factor in determining the conditions for the action of specific phospholipases in membranes.

The authors are grateful to colleagues Drs Coleman, Michell and Knutton and Mr Low for their contributions both to the data referred to in this paper and for their discussion of the structural implications. These studies are supported by the Science Research Council.

References (Finean *et al.*)

Bourges, M., Small, D. M. & Dervichian, D. G. 1967 Biophysics of lipidic associations: II. *Biochim. biophys. Acta* **137**, 157–167.

Bretscher, M. S. 1971 A major protein which spans the human erythrocyte membrane. *J. molec. Biol.* **59**, 351–357.

Bretscher, M. S. 1972*a* Asymmetrical lipid bilayer structure for biological membranes. *Nature New Biol.* **236**, 11–12.

Bretscher, M. S. 1972*b* Phosphatidyl ethanolamine: differential labelling in intact cells and cell ghosts of human erythrocytes by a membrane impermeable reagent. *J. molec. Biol.* **71**, 523–528.

Coleman, R., Finean, J. B., Knutton, S. & Limbrick, A. R. 1970 A structural study of the modification of erythrocyte ghosts by phospholipase C. *Biochim. biophys. Acta* **219**, 81–92.

Colley, C. M., Zwaal, R. F. A., Roelofson, B. & van Deenen, L. L. M. 1973 Lytic and non-lytic degradation of phospholipids in mammalian erythrocytes by pure phospholipases. *Biochim. biophys. Acta* **307**, 74–82.

Finean, J. B. 1972 The development of ideas on membrane structure. *Sub-Cell. Biochem.* **1**, 363–373.

Finean, J. B., Coleman, R., Green, W. G. & Limbrick, A. R. 1966 Low-angle X-ray diffraction and electron-microscope studies of isolated cell membranes. *J. Cell Sci.* **1**, 287–296.

Green, J. R. & Green, C. 1973 The enrichment of erythrocyte membranes and phosphatidylcholine dispersions with cholesterol. *Biochem. Soc. Trans.* **1**, 365–368.

Horwitz, C., Krut, L. & Kaminsky, L. 1971 Cholesterol uptake by egg-yolk phosphatidylcholine. *Biochim. biophys. Acta* **239**, 329–336.

Knutton, S., Finean, J. B., Coleman, R. & Limbrick, A. R. 1970 Low-angle X-ray diffraction and electron-microscope studies of isolated erythrocyte membranes. *J. Cell Sci.* **7**, 357–371.

Ladbrooke, B. D., Williams, R. M. & Chapman, D. 1968 Studies on lecithin–cholesterol–water interactions by differential scanning calorimetry and X-ray diffraction. *Biochim. biophys. Acta* **150**, 335–340.

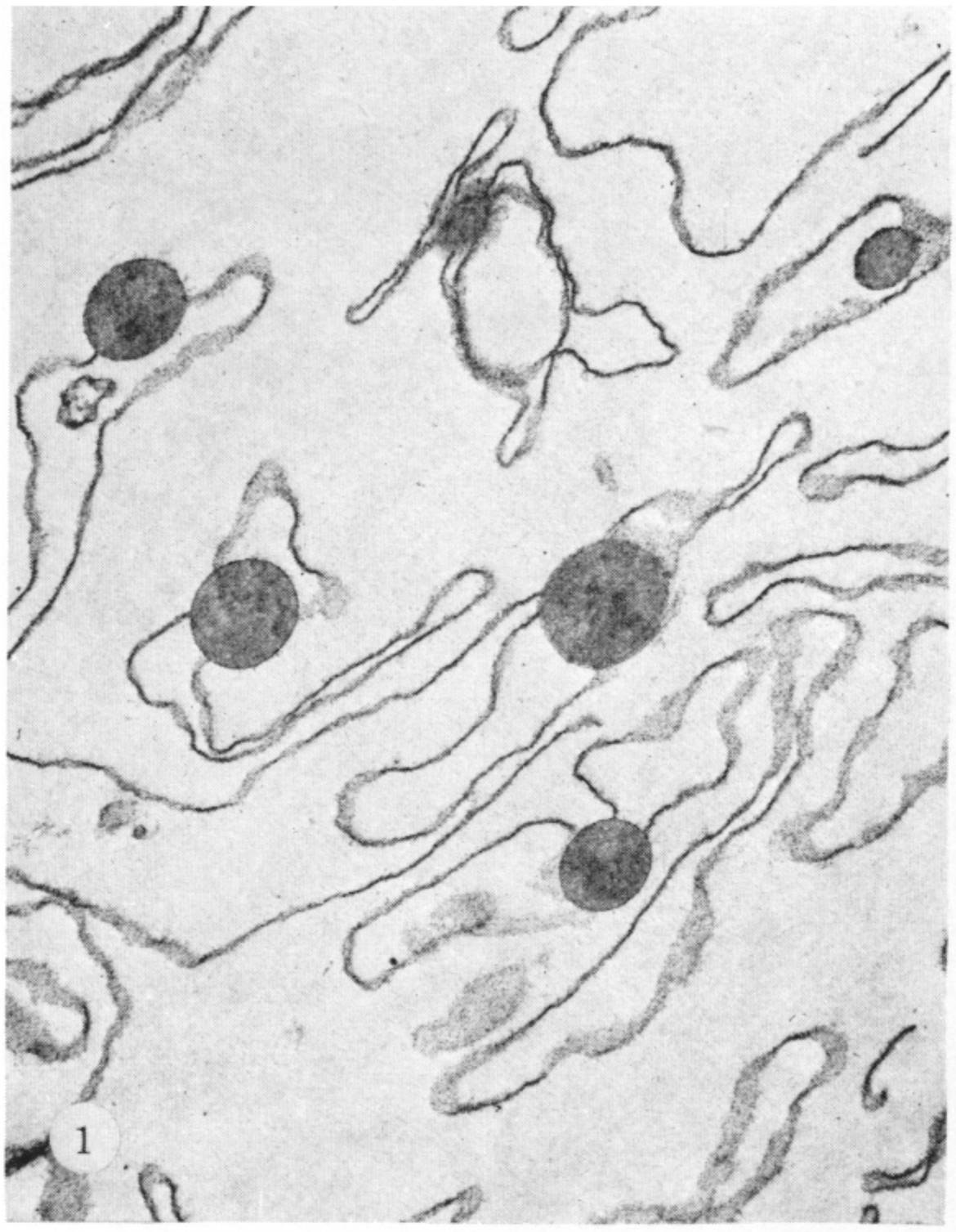
Limbrick, A. R. & Knutton, S. 1971 A structural study of the modification of erythrocyte membranes by phospholipase C. Abstracts of Papers, Eleventh Annual Meeting of the American Society for Cell Biology. p. 168.

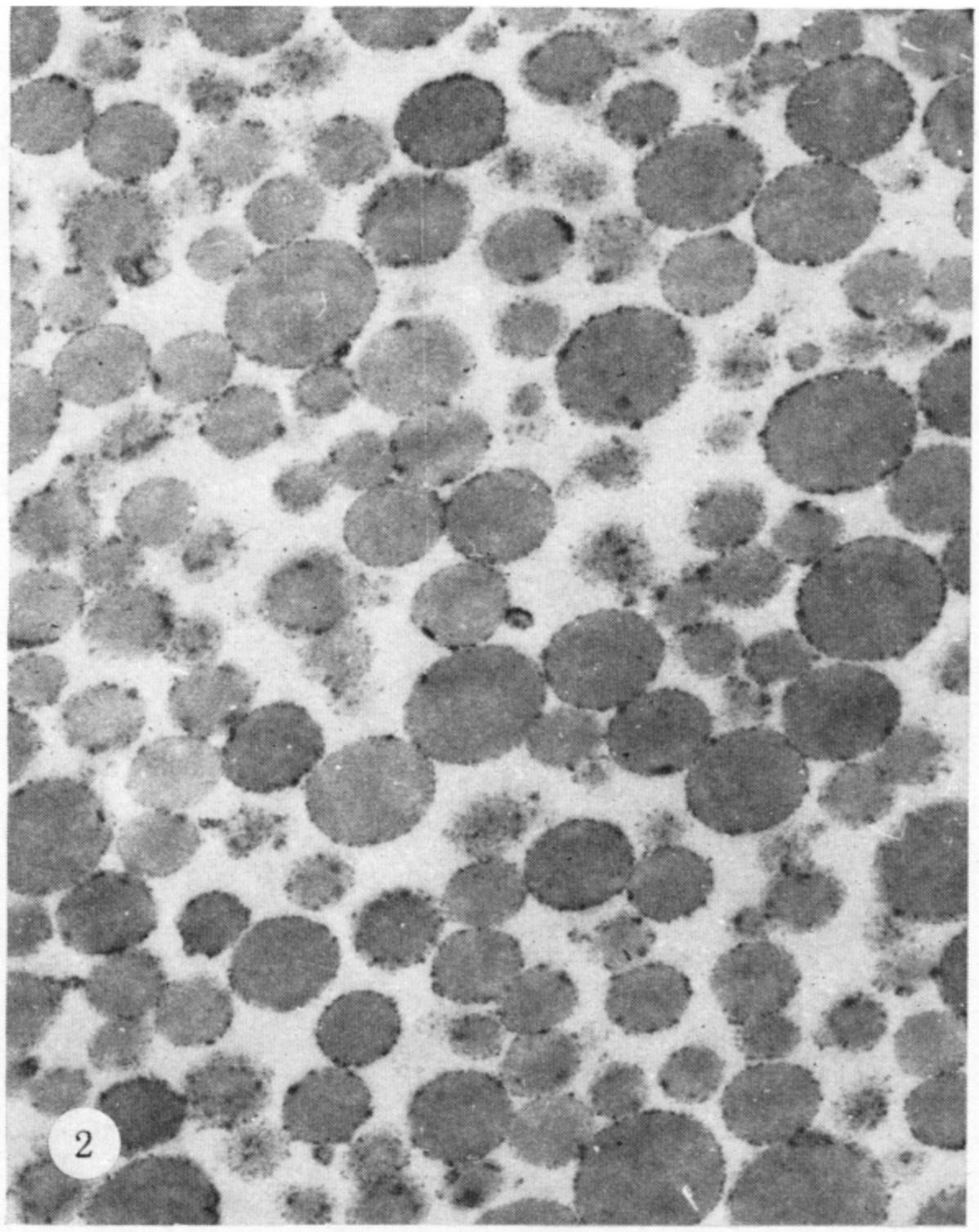
Low, M. J., Limbrick, A. R. & Finean, J. B. 1973 Phospholipase C (*B. cereus*) acts only at the inner surface of the erythrocyte membrane? *F.E.B.S. Lett.* **34**, 1–4.

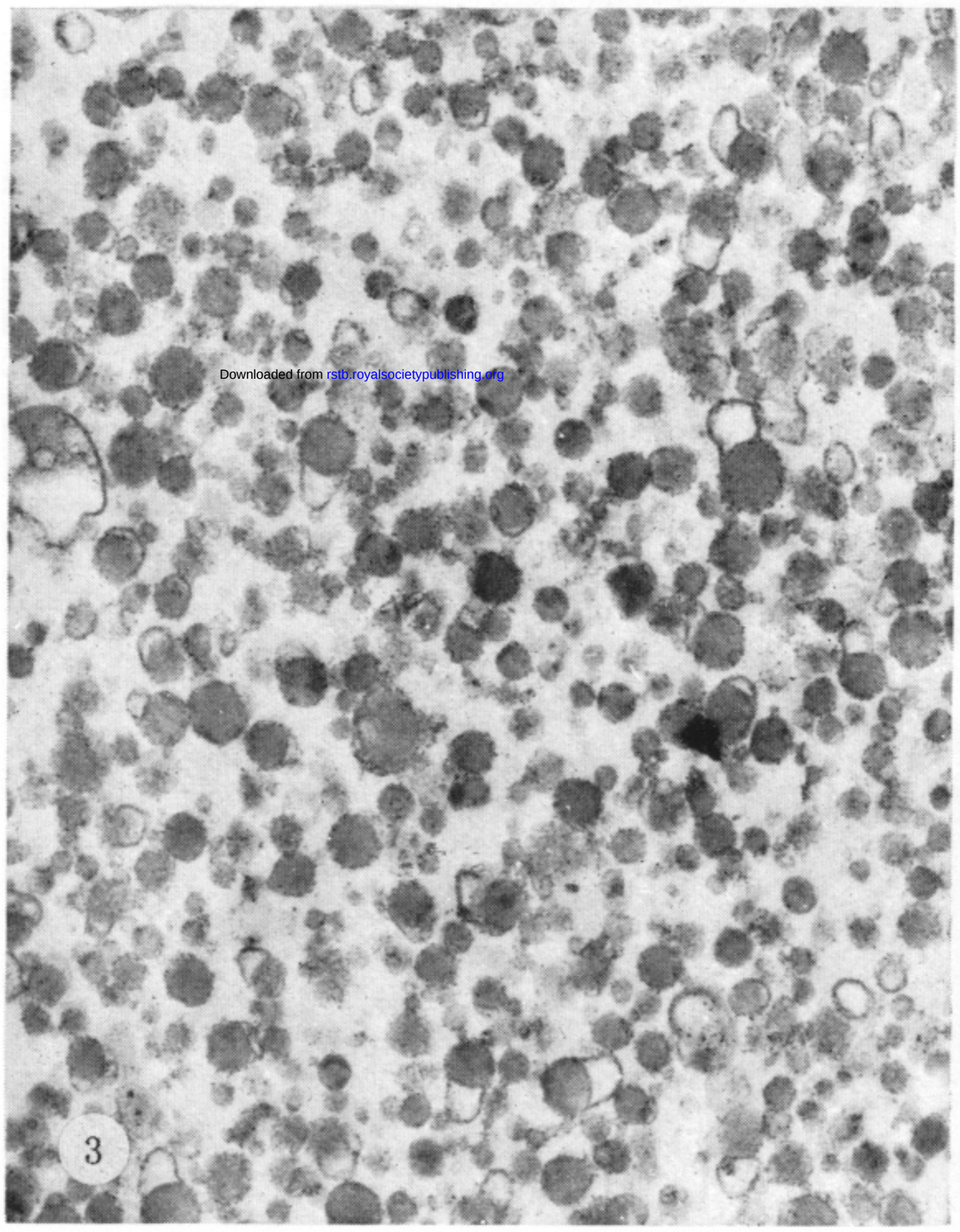
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Michell, R. H., Coleman, R. & Finean, J. B. 1973 Hydrolysis of 1,2-diglyceride by membrane-associated lipase activity during phospholipase C treatment of membranes. *Biochim. biophys. Acta* **318**, 306–312.
Sabban, E., Laster, Y. & Loyter, A. 1972 Resolution of haemolytic and hydrolytic activities of phospholipiase A preparation from *Clostridium perfringens*. *Eur. J. Biochem.* **28**, 373–380.
Segrest, J. P., Kahane, I., Jackson, R. L. & Marchesi, V. T. 1973 Major glycoprotein of the human erythrocyte membrane: evidence for an amphiphilic molecular structure. *Archs Biochem. Biophys.* **155**, 167–183.
Woodward, C. B. & Zwaal, R. F. A. 1972 The lytic behaviour of pure phospholipases A_2 and C towards osmotically swollen erythrocytes and resealed ghosts. *Biochim. biophys. Acta* **274**, 272–278.
Zahler, P. 1969 *Molecular problems of blood preservation*. Stuttgart: Gustav Fischer Verlag.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES







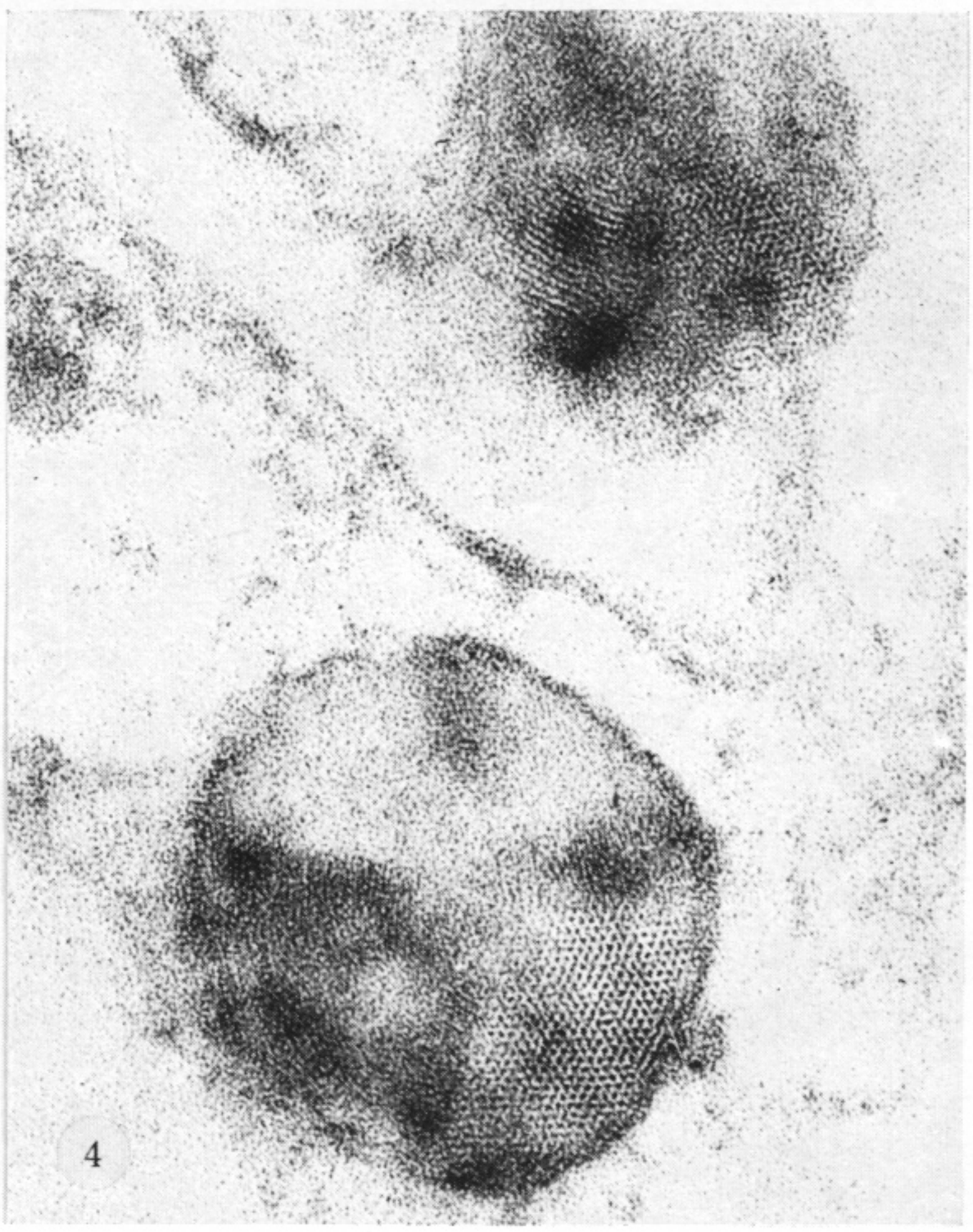
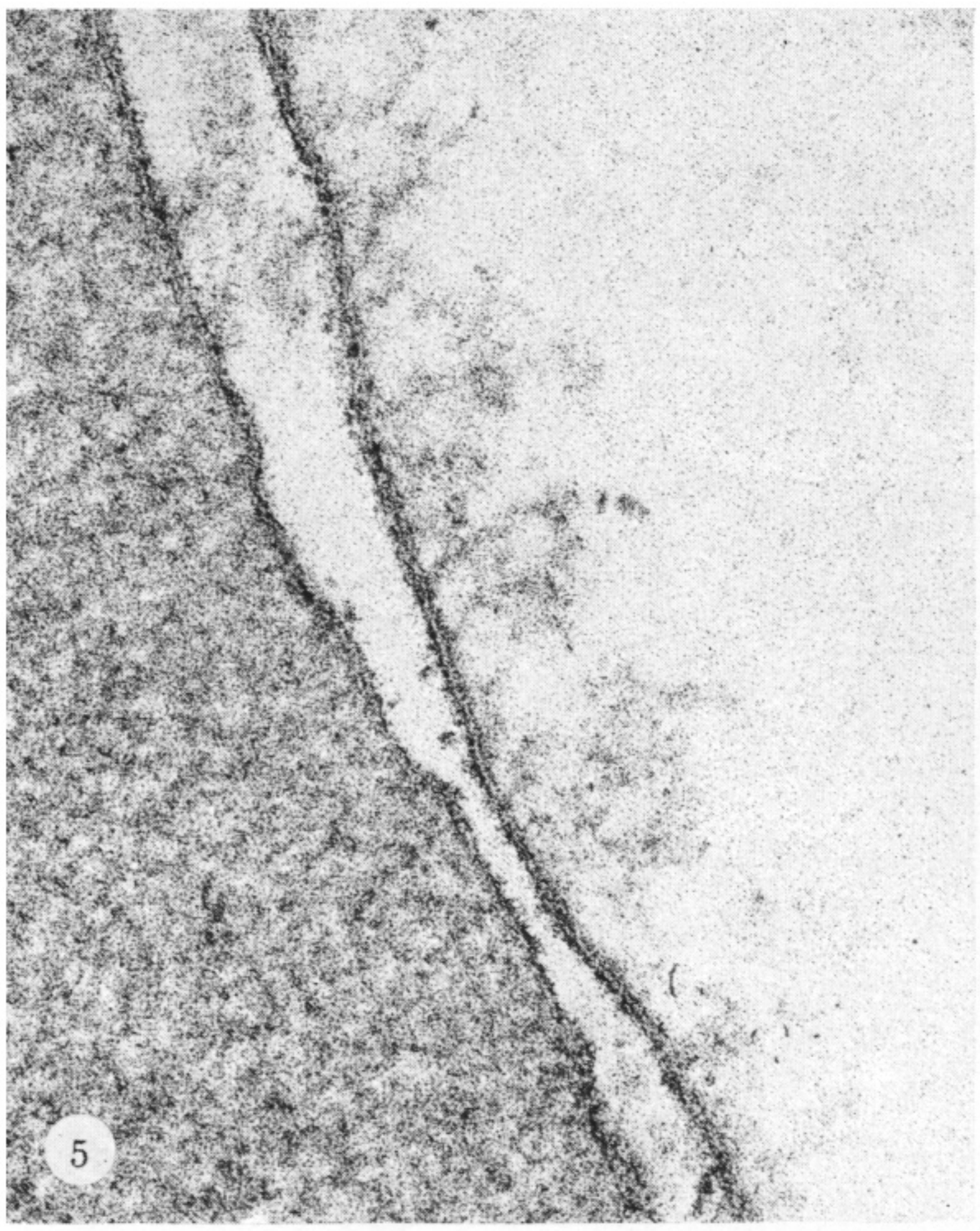


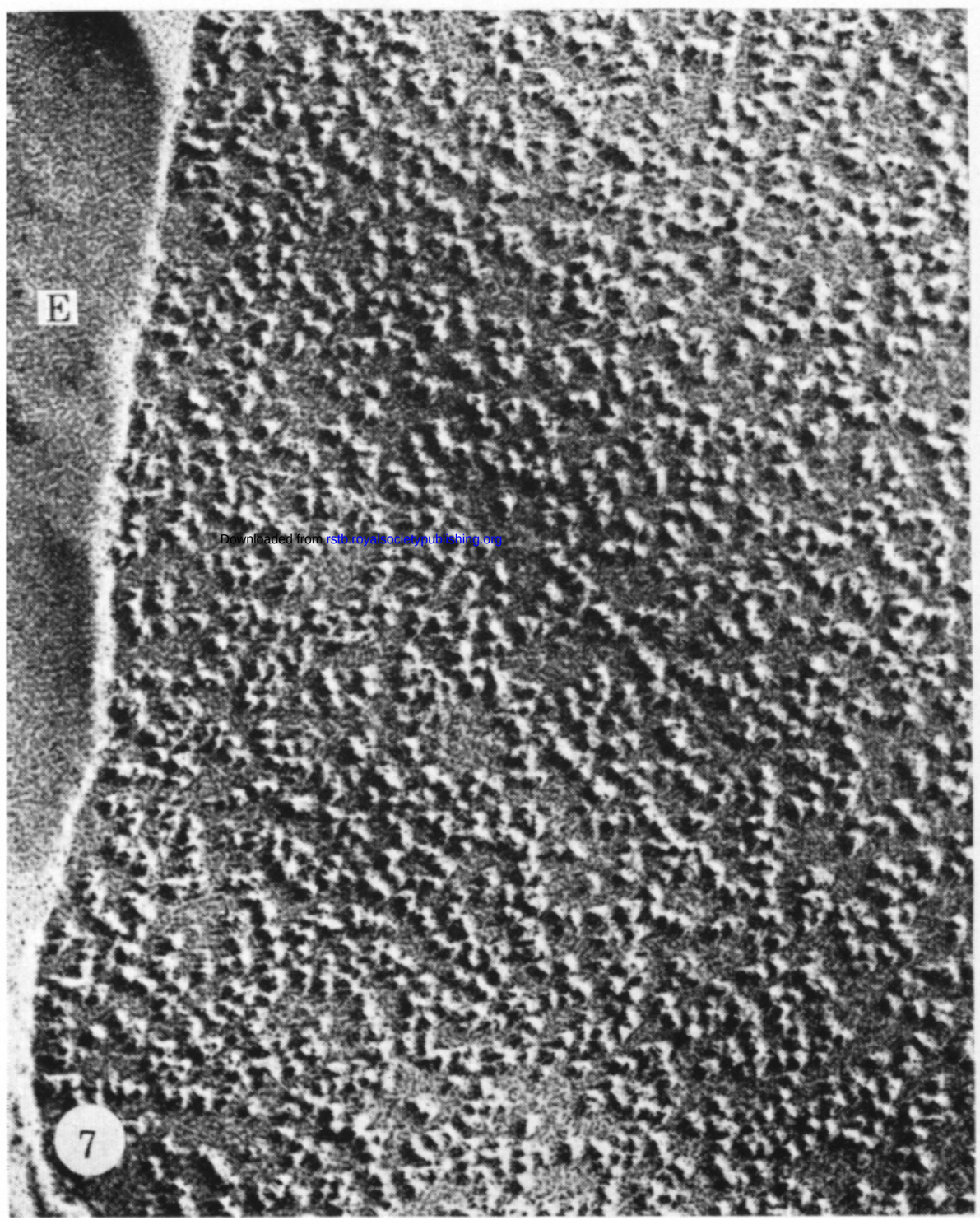
FIGURE 1. Electron micrograph of a section through a haemoglobin-free preparation of human erythrocyte ghosts treated with phospholipase C (*Cl. perfringens*) to maximum phospholipid hydrolysis (~ 70%) (magn. × 30 000).

FIGURE 2. Electron micrograph of a section through a droplet fraction separated from a sonicated preparation of phospholipase C (*Cl. perfringens*) – treated human erythrocyte ghosts (magn. × 30 000).

FIGURE 3. Electron micrograph of a section through a phospholipase C (*Cl. perfringens*)-treated aqueous dispersion of lipid extracted from human erythrocyte ghosts (magn. × 30 000).

FIGURE 4. Electron micrograph of a section through structured droplets in a phospholipase C (*Cl. perfringens*)-treated preparation of pig erythrocyte ghosts (magn. × 160 000).





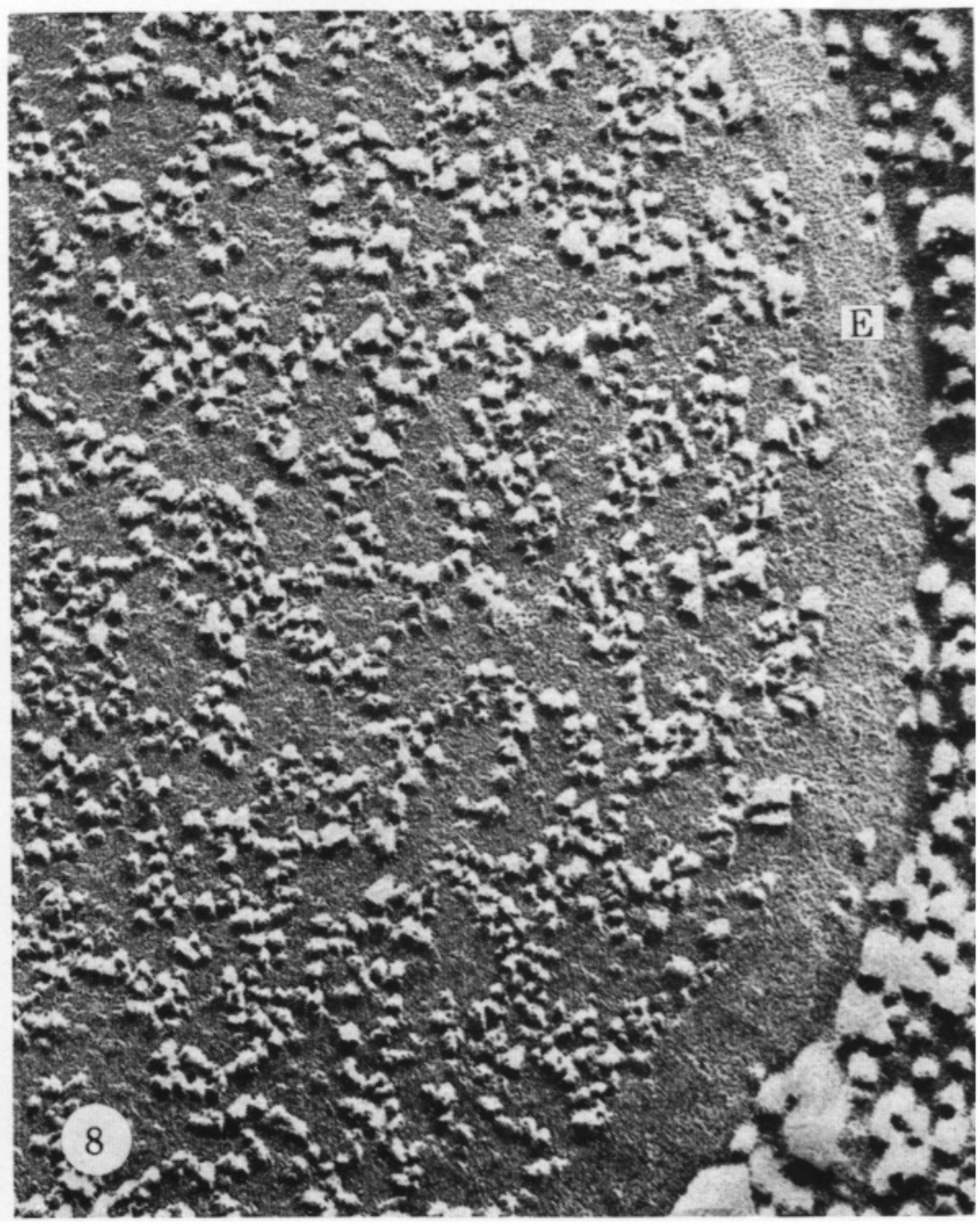


FIGURE 5. Electron micrograph of a section through a sample of human erythrocyte ghosts prepared in 80 mosmol/l buffer and left at room temperature for a few minutes to allow a proportion of the ghosts to reseal before treatment with phospholipase C (*B. cereus*). The ghost on the left had resealed and appeared not to have been modified by the phospholipase C. The ghost on the right carried droplets such as those illustrated in figure 1 which are indicative of phospholipid hydrolysis. (Magn. ×130 000.)

FIGURE 6. A selected area showing close-packed membranes in an electron micrograph of a section through a preparation of pig erythrocyte ghosts which had been haemolysed in less than 3 mosmol buffer and centrifuged at 500 000 *g* for 100 min (Magn. ×110 000).

FIGURES 7 and 8. Electron micrographs of freeze-etched samples of human erythrocyte ghosts. In each case the direction of shadowing is from below. E indicates the etched outer face of the membrane. The main area represents the A fracture face. Figure 7, control preparation; figure 8, preparation modified by mild phospholipase C (*Cl. perfringens*) treatment.