J. Membrane Biol. 2, 180–191 (1970)

Membrane Structure: Morphological and Chemical Alterations in Phospholipase-C-Treated Mitochondria and Red Cell Ghosts

ABRAMO C. OTTOLENGHI and MICHAEL H. BOWMAN

Departments of Medical Microbiology and Anatomy, College of Medicine, The Ohio State University, Columbus, Ohio 43210

Received 7 January 1970

Summary. Phospholipase C from *Bacillus cereus* was used as a tool for the study of membrane structure. Interference and electron microscopic examination of isolated rat kidney mitochondria and human red blood cell ghosts incubated in the presence of the enzyme revealed that there were discrete areas of digestion on the membranes. These areas were distributed over the surface of the membrane but did not coalesce. These data are interpreted as giving support to the membrane model which postulates that protein does not cover the entire phospholipid layer of the membrane, but that there are areas of the membrane in which the phospholipid polar groups are in direct contact with the outside milieu.

The molecular model of membrane structure proposed by Davson and Danielli [1] and modified by Robertson [14] has recently been challenged [4, 6]. Lenard and Singer [7] and Wallach and Zahler [17] have independently proposed similar models, both of which differ from the Davson-Danielli-Robertson model in the arrangement of the phospholipid molecules. In both of these new models, the polar heads of the phospholipid molecules are exposed to the external aqueous phase. This is in contradistinction to the Davson-Danielli-Robertson model in which the bimolecular lipid leaflet is uniformly covered on both the internal and external surfaces by extended polypeptide chains.

In support of this new model, Lenard and Singer [8] have reported that red blood cell (RBC) ghosts treated with phospholipase C from *Clostridium perfringens* lost 68 to 74% of the total membrane phosphorus. These authors also remarked that the size of the ghosts markedly decreased and that they could not observe by phase-contrast microscopy any fragmentation of the membrane itself. Further evidence comes from Finean and Martonosi [3] who found that hydrolysis of muscle microsomes by this enzyme caused an

accumulation of isolated electron-dense blebs on the membranes. Analysis of the material contained in these blebs revealed that diglyceride was the primary constituent. Since phospholipase C specifically catalyzes the hydrolysis of phospholipids to diglycerides and water-soluble phosphorylated amines [11], the data support the concept of exposed phospholipids on the external membrane surface.

In this paper, morphologic data are presented which support the postulates of Lenard and Singer [7] and of Wallach and Zahler [17].

Materials and Methods

Materials

Mitochondria. The normal rat kidney mitochondria used in this study were isolated and the protein content was determined according to Scarpelli [15].

Red Cell Membrane. The RBC ghosts were prepared from human type 0 cells by a modification of the method of Dodge, Mitchell and Hanahan [2], using 20 mosm phosphate buffer, pH 7.4. After the final wash, the ghost pellet was suspended in 50 μM N–2–hydroxyethylpiperazine–N′–2–ethane sulfonic acid (HEPES) buffer (supplied by Calbiochem, Los Angeles, Calif.), pH 7.4, and 1.5 mM with respect to NaCl. After 30 min, the suspension was again centrifuged for 20 min at $32{,}000 \times g$, and the pellet was resuspended in 10 ml of the HEPES buffer.

Phospholipase C. The phospholipase C from *C. perfringens* was obtained from General Biochemical Corp. (Chagrin Falls, Ohio). The phospholipase C from *Bacillus cereus* was prepared and purified according to the method of Ottolenghi [13].

For these experiments the 75% ethanol fraction was used. Purity criteria for this fraction were the absence of protease, nucleotidase, and o-phenanthroline-insensitive activity. When tested against rabbit antiserum prepared against the crude enzyme, a single band of precipitation appeared (Fig. 1). Protein determinations of the enzyme were done using the 280/260 nm optical density method according to Warburg and Christian [18].

Incubation Systems

Mitochondria. A 1.25-ml sample of mitochondrial suspension (4 mg protein) was incubated for 30 min with 25 μg (as protein) of *B. cereus* phospholipase C in a total volume of 2.5 ml made 0.25 M with respect to sucrose. Controls consisted of similar systems without the added phospholipase C or with the phospholipase C and/or o-phenanthroline (1 mg/ml final concentration) to inhibit the action of the enzyme [13].

Erythrocyte ghosts. A 4-ml portion of the 1% ghost suspension was incubated with 1 mg (as protein) of the partially purified *B. cereus* phospholipase at 37 °C for 30 min. Controls consisted of incubation mixtures containing no phospholipase, phospholipase and o-phenanthroline (1 mg/ml final concentration), and o-phenanthroline alone.

Lipophilic Staining. To 1 ml of the RBC ghost suspension, either without treatment or with phospholipase C digestion, was added 0.1 ml of sudan black (saturated solution in 70% ethanol) or 0.1 ml of oil red 0 (0.3% in 99% isopropanol). After 10-min incubation at room temperature, the suspension was observed under bright-field conditions.

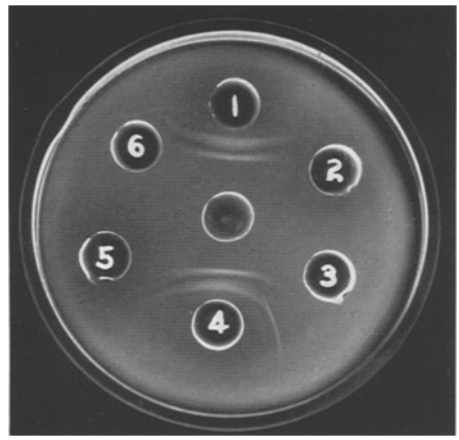

Fig. 1. Immunodiffusion pattern observed with purified phospholipase C from *B. cereus* vs. a rabbit serum prepared against crude enzyme. The crude antigen is contained in the central well, and the purified antigen is in well *3*. Wells *2* and *5* contain normal rabbit serum; well *6* is a saline control. The antibody to the crude enzyme is in wells *1* and *4*

Preparation for Electron Microscopy

Mitochondria. After incubation the mitochondria were centrifuged at 10,000× *g* for 20 min and then prepared for electron microscopy as described by Scarpelli [15]. Maraglass was used for embedding. The specimens were stained with uranyl acetate and lead citrate. After sectioning the material was observed in an RCA EMU-3 electron microscope.

Erythrocyte Ghosts. After incubation the cells were centrifuged at 32,000× *g* for 20 min. The pellet was resuspended in cold 2.5% glutaraldehyde buffered at pH 7.4 with phosphate buffer. After 1-hr fixation, the membranes were centrifuged out and the supernatant discarded. The pellet formed was washed with Sorenson's buffer, pH 7.4, and postfixed in 1% osmium tetroxide buffered with the same phosphate buffer. After dehydration the specimen was embedded in Dow Epoxy resin with the No. 1 mixture described by Lockwood [9]. Sections were cut with a Porter-Blum MT-2 ultramicrotome and examined with an RCA EMU-3 or a JEM 100-B electron microscope.

Phosphorus Determinations

Subsequent to the period of incubation, two appropriate samples (usually 1.0 ml) of the mixture were centrifuged separately. The supernatants were decanted and kept. The pellets were washed twice with 2 ml of double-distilled water and the washes added to the supernatants from the first centrifugation. After the samples (pellets and supernatants) were dried, 1 ml of 70% perchloric acid was added and, after oxidation, phosphorus was determined by the method of Lowry, Roberts, Leiner, Wu and Farr [10].

Results

Light and Electron Microscopy

Mitochondria. The results obtained in one of three experiments can be seen in Fig. 2. It is apparent that there are discrete noncoalescing electron-

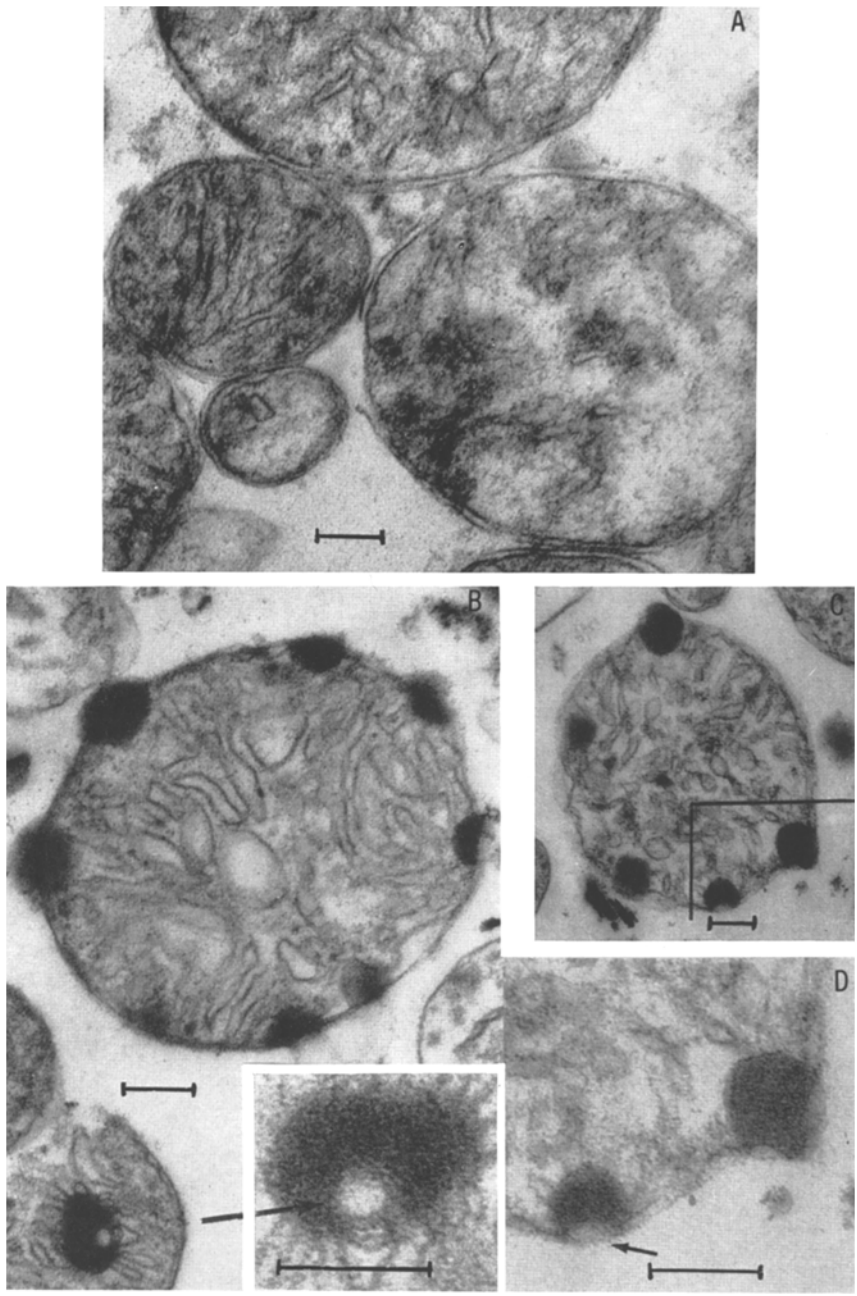

Fig. 2. (A) Control preparation of rat kidney mitochondria. (B) Section of rat kidney mitochondria after digestion with *B. cereus* phospholipase C. Insert: arrow shows clear area in center of electron-dense region. (C) Section of same pellet as (B), indicating multiply digested membrane and establishing reference for (D). (D) Detail of (C) showing invagination (arrow) of the electron-dense area. Scale: 0.2 μ

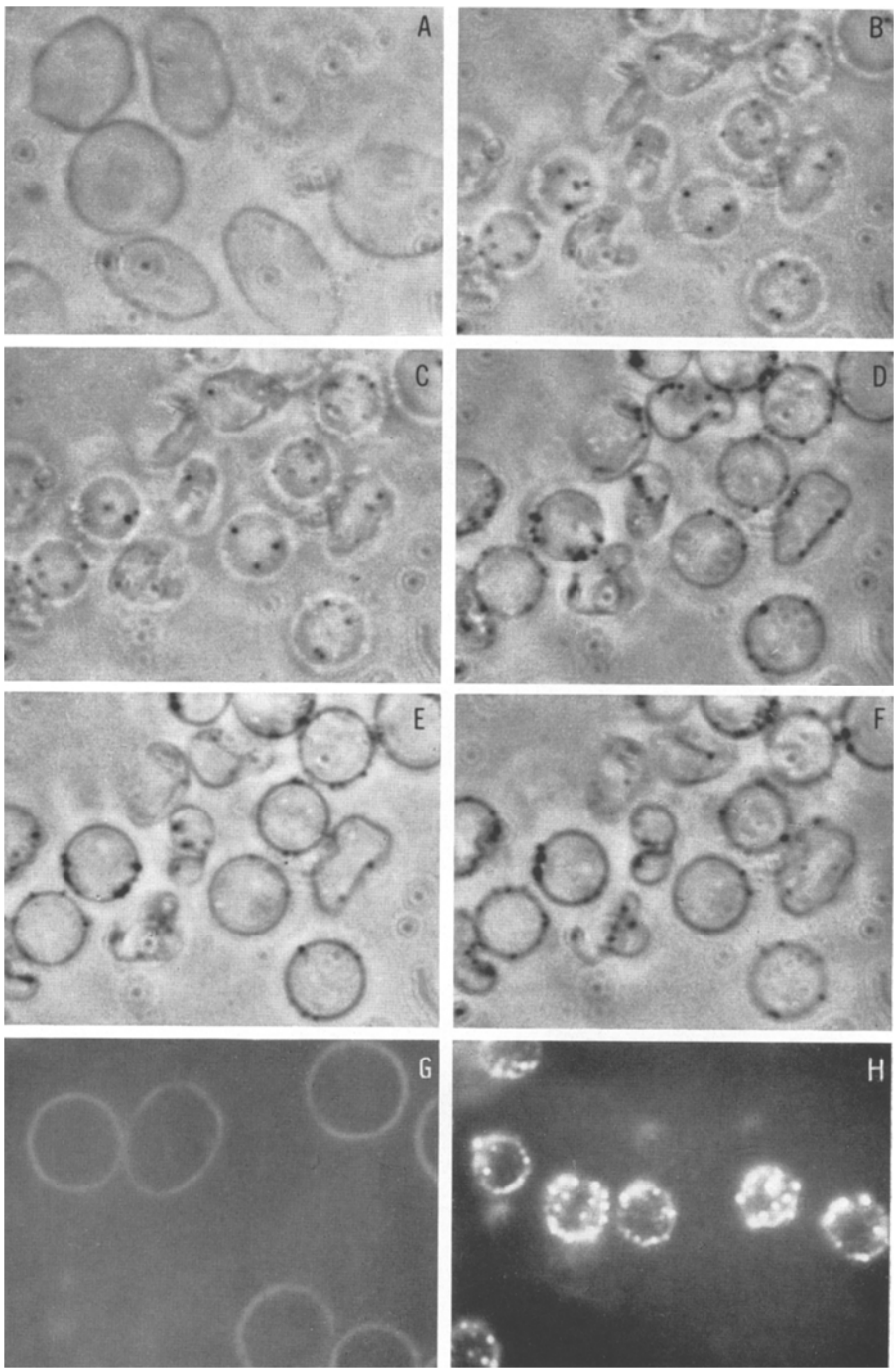

Fig. 3. (A) Control preparation of human RBC ghosts under phase-contrast illumination. (B)–(F) Through-focus series of suspensions of RBC ghosts digested with phospholipase C from *B. cereus*, indicating discrete areas of light diffraction and distribution of

dense areas in array around the periphery of the mitochondrion. The distribution and frequency of these areas differed from section to section. The figures shown were chosen to demonstrate sections with a maximal number of electron-dense areas on one mitochondrial section.

Depending on the plane of the cut, some of the electron-dense areas showed clear areas in the center (Fig. 2B and insert) or, as in Fig. 2C and D, an invagination. In all cases, however, the electron-dense areas were discrete and did not fuse.

Erythrocyte Ghosts. Digestion of human erythrocyte ghosts resulted in the modifications observed in Fig. 3 which were evident under both phase contrast (Fig. 3B–F) and dark field (Fig. 3H). In both cases, discrete areas of different light-scattering properties appear at the periphery of the membranes. There is, however, no disintegration of the membrane.

The decrease in size of the RBC ghost is also very noticeable. This observation is in agreement with that described by Lenard and Singer [8]. Our results extend the observations reported by Lenard and Singer [8] who did not report these discrete altered areas. In experiments using the phospholipase C from *C. perfringens* rather than the *B. cereus* enzyme, similar results were observed.

Under bright-field microscopy, these ghosts are not visible; however, when a lipophilic dye such as sudan black or oil red 0 was mixed with the enzyme-treated ghosts, there appeared along the faint outline of the ghost itself a number of black or red dots, respectively. These colored dots corresponded to the areas of different light scattering in the indirect-light observations.

Electron microscope observation revealed that, as in the mitochondria, subsequent to enzyme action, there are produced in RBC membranes discrete areas of electron-dense material scattered along the section (Fig. 4B).

Control preparations (Fig. 4A) were all similar, whether obtained from untreated ghosts or from ghosts treated with both the phospholipase and/or o-phenanthroline (Fig. 4C).

Fig. 4D and E show the type of association which has been observed between the droplet and the membrane. In Fig. 4E particularly, it is very

such areas throughout the ghost with no destruction of the membrane. (G) Control preparation of RBC ghosts seen with dark-field illumination. (H) Digested ghosts under dark field.

The difference in size between control preparations (A and G) and the treated preparations can be easily seen. × 1,750 (by reproduction reduced to 9/10)

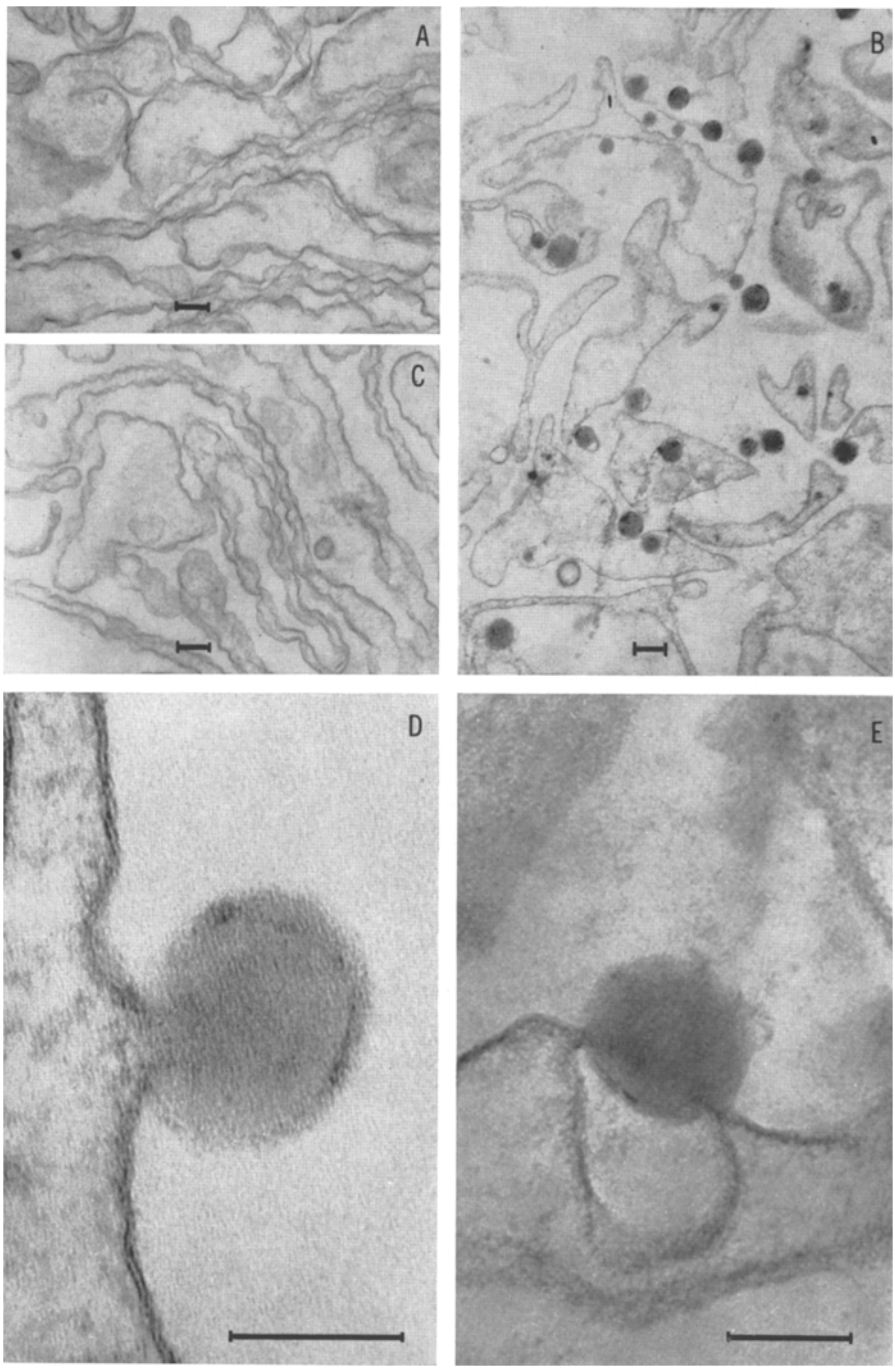

Fig. 4. (A) Control preparation of human RBC ghosts fixed in glutaraldehyde, postfixed in osmium tetroxide, and stained with uranyl acetate and lead citrate. ×8,000 (by reproduction reduced to 9/10). (B) Human RBC ghosts treated with phospholipase C from

tempting to interpret the droplet as moving out of the pocket pictured but being retained attached to the membrane by forces at the limit of the digested area.

Higher-magnification pictures of the electron-dense areas (Fig. 5) showed that these droplet-like structures exhibited lamellar arrangements which are more or less evident depending on the plane of the cut.

Phosphorus Release

The results of two experiments are described in the Table. These results are in agreement with the values of 68 to 75% given by Lenard and Singer [8] for the release of phosphorus from red cell ghosts.

Table. *Phosphorus determinations subsequent to phospholipase digestion of human RBC ghosts*

Additions	Phosphorus (average of duplicate determinations; μg)			
	Exp. 1[a]		Exp. 2[b]	
	Pellet	Supernatant	Pellet	Supernatant
RBC ghosts	7.3	0	5.60	0
Phospholipase	(no pellet)	1.2	(no pellet)	0.6
RBC ghosts + phospholipase	2.5	5.65	1.5	4.4
Phosphorus released (corrected for phospholipase blank)	4.45		3.8	

[a] A 4-ml sample of a 1% RBC ghost suspension was incubated with 0.1 ml (1 mg protein) of phospholipase C for 30 min at 37 °C. After incubation, duplicate 1-ml samples of the mixture were centrifuged, and the supernatant and pellet were separated. The pellet was washed twice with distilled water, and phosphorus determinations were performed on the pellet and the supernatant and washes.

[b] Procedure was as in Exp. 1 except that 0.6-ml samples were used.

B. cereus and fixed as in (A), showing electron-dense areas. × 8,000 (by reproduction reduced to 9/10). (C) Same as (B), but o-phenanthroline (1 mg/ml) was added at the beginning of incubation. Note that the membrane resembles the control (A) in that no electron-dense areas appear. (D) – (E) Same as (B), but details of attachment of the electron-dense areas to the membrane are shown × 50,000 and 35,000 respectively (by reproduction reduced to 9/10)

All observations obtained with an RCA EMU-3 electron microscope. Scale: 0.5 μ

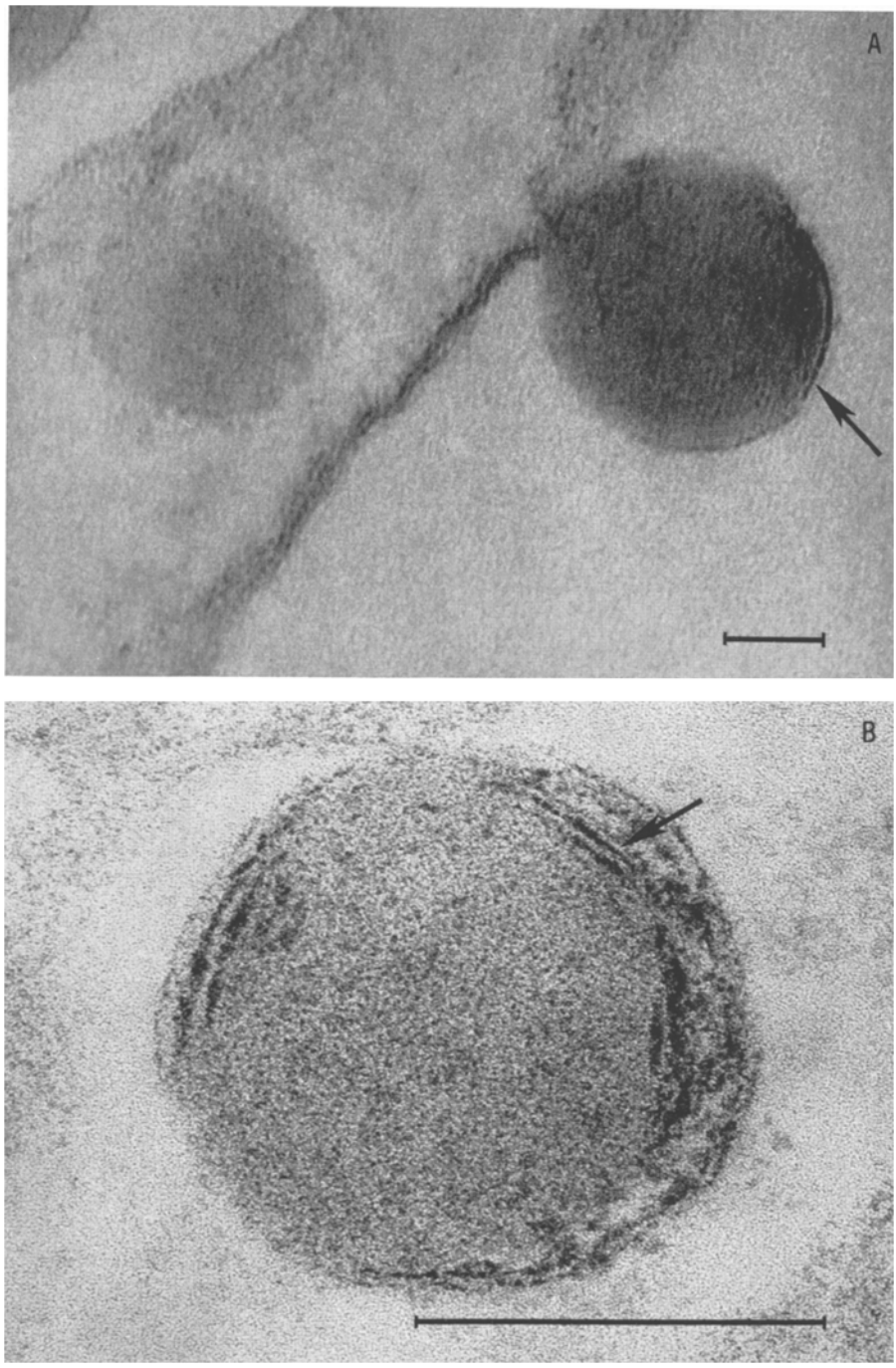

Fig. 5. (A) Enlargement of electron-dense area appearing after digestion of human RBC ghosts with *B. cereus* phospholipase C. Preparation as in Fig. 3. Arrow indicates limiting denser area. × 72,000 (by reproduction reduced to 9/10). (B) Enlargement of electron-dense area prepared as in (A), with arrow indicating series of denser areas possibly showing smectic mesophase arrangement. These areas measure from line to line about 100 A. × 300,000 (by reproduction reduced to 9/10). Scale: 0.2 μ

Discussion

The use of phospholipase C for the elucidation of membrane structure has in the past been carried out with enzyme preparations where no attempts had been made even to purify them partially, to test for activities other than phospholipase activity or to control chemically the enzymatic activity.

In this study, purification of the enzyme used, examination for nucleotidase and protease activity, as well as chemical control of the enzymatic activity, give more assurance that the results observed are indeed due to the phospholipase activity.

Although the immunological homogeneity of the enzyme is in itself no guarantee of its functional homogeneity, the use of o-phenanthroline, which has been shown to inhibit specifically the action of the *B. cereus* phospholipase C [13], has permitted a demonstration of the fact that no changes occur in the presence of this chelating agent.

This study, therefore, establishes with greater assurance than ever before that the results observed are indeed produced by the activity of the phospholipase C, and that there are no artifacts produced by other activities of the preparation.

With the classical Davson-Danielli [1] membrane model, one postulates that in biological membranes the phospholipids are arranged between two layers of protein with the hydrophilic groups proximal to the proteins. In practice, electron microscopists have interpreted the "double track" figure that is often seen in membranes as supporting this theory.

From the observations reported here, however, it would appear that the phospholipids must be in contact at some points with the aqueous medium and that at these points the polar groups are not bound to the protein. If this were not the case, it would be difficult for the phospholipase to penetrate to the site of the substrate. Ottolenghi has also shown [12] that substances which interact with the polar group of phospholipids interfere with the action of the phospholipase used in this study.

These data giving morphological evidence that there are circumscribed areas on membranes at which the phospholipids are accessible to the phospholipase, and that they are in intimate contact with the outside, are in general accord with the membrane models postulated by Lenard and Singer [7], as well as by Wallach and Zahler [17].

The question must remain open as to whether the droplets represent the accumulation of diglyceride following the *in situ* digestion of phospholipids or whether they represent the migration of reaction products from other sites on the membrane.

On one hand, it can be argued that the non-coalescing nature of the droplets speaks for morphological characteristics which keep them separate, whereas the relatively large size of the droplets can be invoked to argue that there is more lipid present there than could be present in such a small portion of membrane.

Assuming the migration of lipid to a given spot to form a droplet, the fact must still remain that phosphate groups must be accessible to the enzyme through some portal or across the whole membrane. Neither of these possibilities is in accordance with the Davson-Danielli model for membranes.

Grossly, the results reported here for mitochondria are in accordance with those seen in electron micrographs published by Grossman, Heitkamp and Sackton [5] and by Strunk, Smith and Blumberg [16] with the phospholipase C from *C. perfringens*. The results reported by these authors show that the mitochondria develop discrete electron-dense areas after incubation (both *in vitro* and *in vivo*) with phospholipase C. The main difference in our results is that in our case there are more electron-dense areas on the periphery of the mitochondria. In both cases, however, the electron-dense areas are clearly circumscribed.

The blebs observed in the indirect-light microscopy studies of the RBC ghosts are probably diglyceride. It has not been possible to establish this point since attempts to extract the ghosts with the usual lipid solvents have resulted in the collapse of the structure so that no relationships could be established. The fact that lipophilic dyes are taken up by these areas would agree with this assumption.

It is interesting to note that the release of any of these droplets into the supernatant has not been observed and that sonic disintegration fragmented the remaining membrane without releasing a significant number of droplets into the supernatant. It would appear that some very strong forces keep them attached to the membrane.

The significance of the invagination seen in the electron-dense spots subsequent to digestion of the mitochondria or of the lamellar-like figures seen in the electron-dense spots after enzymatic digestion of the erythrocyte membranes is not clear at this point, although the latter might represent a smectic mesophase arrangement of the diglyceride produced.

This research was supported by U.S. Public Health Service grant AI 6601-01 from the National Institute of Allergy and Infectious Diseases and by National Institutes of Health General Research Support grant FR-5409-9 to this institution.

References

1. Danielli, J. F., Davson, H. A. 1935. A contribution to the theory of permeability of thin films. *J. Cell. Comp. Physiol.* **5**:495.
2. Dodge, J. T., Mitchell, C., Hanahan, D. J. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**:119.
3. Finean, J. B., Martonosi, A. 1965. The action of phospholipase C on muscle microsomes: a correlation of electron microscope and chemical data. *Biochim. Biophys. Acta* **98**:547.
4. Green, D. E., Perdue, J. F. 1966. Membranes as expressions of repeating units. *Proc. Nat. Acad. Sci.* **55**:1295.
5. Grossman, I. W., Heitkamp, D. H., Sackton, B. 1967. Morphologic and biochemical effects of *Clostridium perfringens* alpha toxin on intact and isolated skeletal muscle mitochondria. *Amer. J. Pathol.* **296**:77.
6. Korn, E. D. 1966. Structure of biological membranes. *Science* **153**:1491.
7. Lenard, J., Singer, S. J. 1966. Protein conformation in cell membrane preparations as studied by optical rotatory dispersion and circular dichroism. *Proc. Nat. Acad. Sci.* **56**:1828.
8. — — 1968. Structure of membranes: reaction of red blood cell membranes with phospholipase C. *Science* **159**:738.
9. Lockwood, W. R. 1964. A reliable and easily sectioned epoxy embedding medium. *Anat. Rec.* **150**:129.
10. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M.-L., Farr, A. L. 1954. The quantitative histochemistry of brain. 1. Chemical methods. *J. Biol. Chem.* **207**:1.
11. Macfarland, M. G. 1948. The biochemistry of bacterial toxins. 2. The enzymatic specificity of *Clostridium welchii* lecithinase. *Biochem. J.* **42**:587.
12. Ottolenghi, A. C. 1967. Cationic antibiotics and phospholipase C as tools in the study of phospholipid structure and function. *Canad. J. Biochem.* **45**:245.
13. — 1969. Phospholipase C. *In:* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press, New York. (*in press*)
14. Robertson, J. D. 1964. Unit membranes: A review with recent new studies of experimental alterations and a new subunit structure in synaptic membranes. *In:* Cellular Membranes in Development. M. Locke, editor. p. 1. Academic Press, New York.
15. Scarpelli, D. G. 1965. Experimental nephrocalcinosis. *Lab. Invest.* **14**:123.
16. Strunk, S. W., Smith, C. W., Blumberg, J. M. 1967. Ultrastructural studies on the lesion produced in skeletal muscle fibers by crude type A *Clostridium perfringens* toxin and its purified alpha fraction. *Amer. J. Pathol.* **296**:89.
17. Wallach, D. F. H., Zahler, P. H. 1966. Protein conformations in cellular membranes. *Proc. Nat. Acad. Sci.* **56**:1552.
18. Warburg, O., Christian, W. 1942. Isolierung und Kristallisation des Gärungsferments Enolase. *Biochem. Z.* **310**:384.