J. Membrane Biol. 6, 81–88 (1971)

In Vitro Excitation of Purified Membrane Fragments by Cholinergic Agonists

IV. Ultrastructure, at High Resolution, of the AcChE-Rich and ATPase-Rich Microsacs

JEAN CARTAUD, E. LUCIO BENEDETTI, MICHIKI KASAI and JEAN-PIERRE CHANGEUX

Laboratoire de Microscopie Electronique, Institut de Biologie Moléculaire, Faculté des Sciences, and Département de Biologie Moléculaire, Institut Pasteur, Paris, France

Received 31 December 1970; revised 12 May 1971

Summary. Membrane fragments rich in acetylcholinesterase (AcChE) or in Na^+-K^+-ATPase are observed under the electron microscope on thin sections after fixation, after negative staining of unfixed material, and after freeze-etching. Both classes of membrane fragments make closed approximately spherical vesicles, or microsacs. The preparation appears to be free from mitochondria, nuclear envelopes or other cytoplasmic contamination. A subunit structure is seen with both kinds of microsacs by freeze-etching and negative staining, but the size, shape and arrangement of the subunits are different in the two classes of membrane fragments. On thin sections, globular repeating units are seen only with the AcChE-rich microsacs; the membrane of the ATPase-rich microsacs shows a classic triple-layered structure.

In the preceding three papers, the functional properties of membrane fragments isolated from the electric organ of *Electrophorus electricus* were presented and extensively discussed. In an effort to correlate these properties with a characteristic structural organization of the membrane, a study of the ultrastructure at high resolution of the acetylcholinesterase (AcChE)-rich and ATPase-rich membrane fragments was initiated. We present here the first results of this study. Using three different techniques, we show that these fragments make vesicles with an approximately spherical shape. Significant differences in the structural organization of the two different classes of microsacs are reported.

Materials and Methods

The microsacs were prepared and purified following the technique described in paper I of this series.

Preparation of the specimens for electron microscopy was carried out in three different ways.

(1) For positive staining, the pellets were fixed for 1 hr at room temperature in 0.1 M phosphate buffer containing 3.5% glutaraldehyde (pH 7.3) and post-fixed for 1 hr in 0.1 M phosphate buffer containing 1% osmium tetroxide (pH 7.3). The material was then embedded in Vestopal W. Thin sections were stained with uranyl acetate and lead citrate according to Reynolds (1963).

(2) A suspension of microsacs in 0.7 M sucrose or after dialysis against 13 mM ammonium acetate overnight at 4° was negatively stained on carbon-coated grids with 1% sodium phosphotungstate (pH 7.3).

(3) For freeze-etching, the unfixed pellets were resuspended in a small amount of 25% glycerol for 1 hr. Small drops of the suspension were mounted on copper discs and frozen rapidly in liquid Freon 22. All the samples were freeze-etched in a Balzers apparatus. The etching time was 1.30 min at −95 °C. During the etching, contamination was prevented by putting the cooled knife on the top of the fractured surface of the specimen. The replicas were made by shadowing with carbon and platinum.

All the specimens were studied with a Phillips EM 300 using a double condenser system. The anticontamination cooling device was routinely used.

Results

Thin Sections of Microsacs

In Fig. 1 are shown two representative areas of AcChE-rich and ATPase-rich microsacs, studied on thin sections. The membrane fragments appear to be free from mitochondrial and nuclear envelope or other cytoplasmic contamination. In the case of the AcChE-rich microsacs, the membrane fragments make, in general, closed vesicles (Fig. 1A). On the other hand, the ATPase-rich fragments are more frequently open and sometimes have a spiral-like shape (Fig. 1C). Moreover, the diversity of size is more pronounced with the ATPase-rich microsacs than with the AcChE-rich ones.

The most striking difference between the two classes of microsacs in thin sections concerns the thickness of the membrane and its apparent structure. The AcChE-rich microsac membrane is 100 Å thick and presents a typical "subunit" structure. Globules are seen within the membrane in both transverse and tangential sections. The electron-lucent areas in the center of the globules have a diameter of 40 Å (Fig. 1B).

The membrane of the ATPase-rich microsacs is thicker (110 Å) and does not show the subunit structure. In transverse section the membrane shows the typical triple-layered structure (Fig. 1D).

Negative Staining

After negative staining, the microsacs embedded in a thin film of sodium phosphotungstate appear to be collapsed. The membrane of the AcChE-rich microsacs looks as if it were formed by an array of globular subunits with a

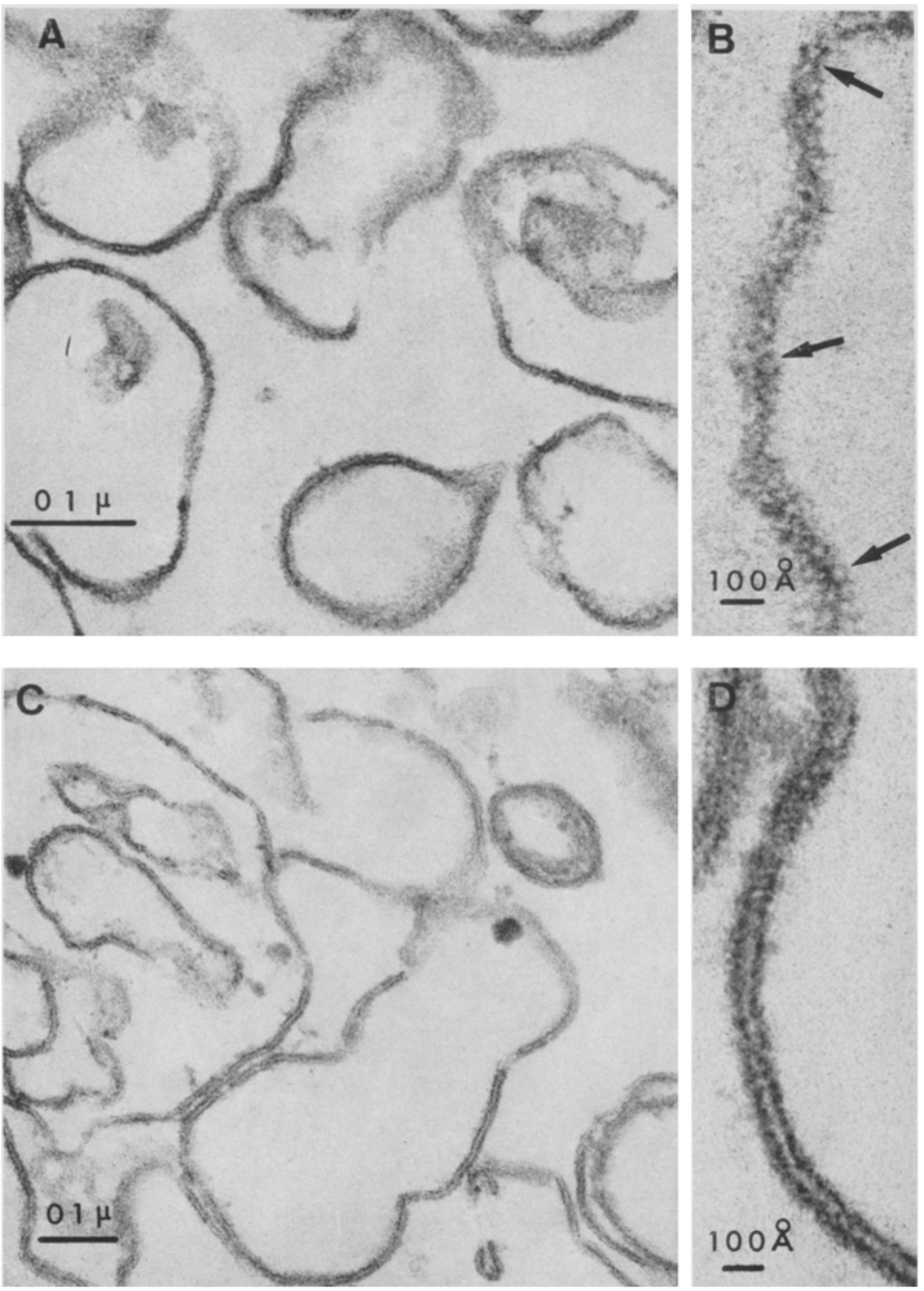

Fig. 1. Thin sections after fixation. (A) Microsacs of the AcChE-rich fraction. Thin section showing that the microsacs appear as closed vesicles having rather regular size. (B) High magnification showing that the membrane element consists of a row of globular repeating units (arrow). (C) Microsacs of ATPase-rich fraction. Thin section showing that the vesicles have a more irregular shape and size. The membrane element is characterized by a triple-layered structure (D)

center-to-center distance of 60 Å (Fig. 2A). The ATPase-rich microsac surfaces are covered by small globular subunits which are particularly visible at the edge of the membrane sheets. These units, with an average diameter of 50–60 Å, are attached to the surface either directly or by a short stem (Figs. 2B & C).

Freeze-Etching

Since the preparations were carried out in the presence of 25% glycerol, most probably there is little etching effect and the exposed surfaces likely correspond to the fractured faces. In Fig. 3A is presented a low-magnification micrograph of the AcChE-rich microsacs having a rather regular size and shape with an average "apparent" diameter of 0.1 μm under the present experimental conditions.

The exposed surfaces are characterized, for the two classes of membrane fragments, by particulate components. Two different kinds of particles are visible in the replicas of the AcChE-rich microsacs. The main membrane framework appears to be made up of globules of 100 or 110 Å in diameter assembled in a rather regular pattern. Globules of a smaller size (80 Å) are seen, dispersed or in small clusters, protruding from the plane of the main membrane framework (Fig. 3B).

Two classes of globules are also seen in the ATPase-rich microsacs. The membrane framework appears to consist of small globules (80 Å in diameter), and, on its surface, clusters of larger globules are observed. In spite of a prolonged etching time (2 min at −95 °C), no surfaces other than those exposed by the fracture have been revealed (Fig. 4).

Conclusions

Electron-microscopic observations of the AcChE-rich and ATPase-rich membrane fragments further strengthen our earlier conclusion that these fragments make closed vesicles or microsacs. In addition, they show that there is little contamination of our preparations by nuclear, mitochondrial or cytoplasmic elements. The two classes of membrane fragments which were separated on the basis of their different enzyme content present striking morphological differences.

On thin sections, the membrane element of the AcChE-rich microsac consists of globular repeating units, whereas the ATPase-rich microsacs show a typical triple-layered structure. By freeze-etching and negative staining, globular units are seen with both classes of membrane fragments.

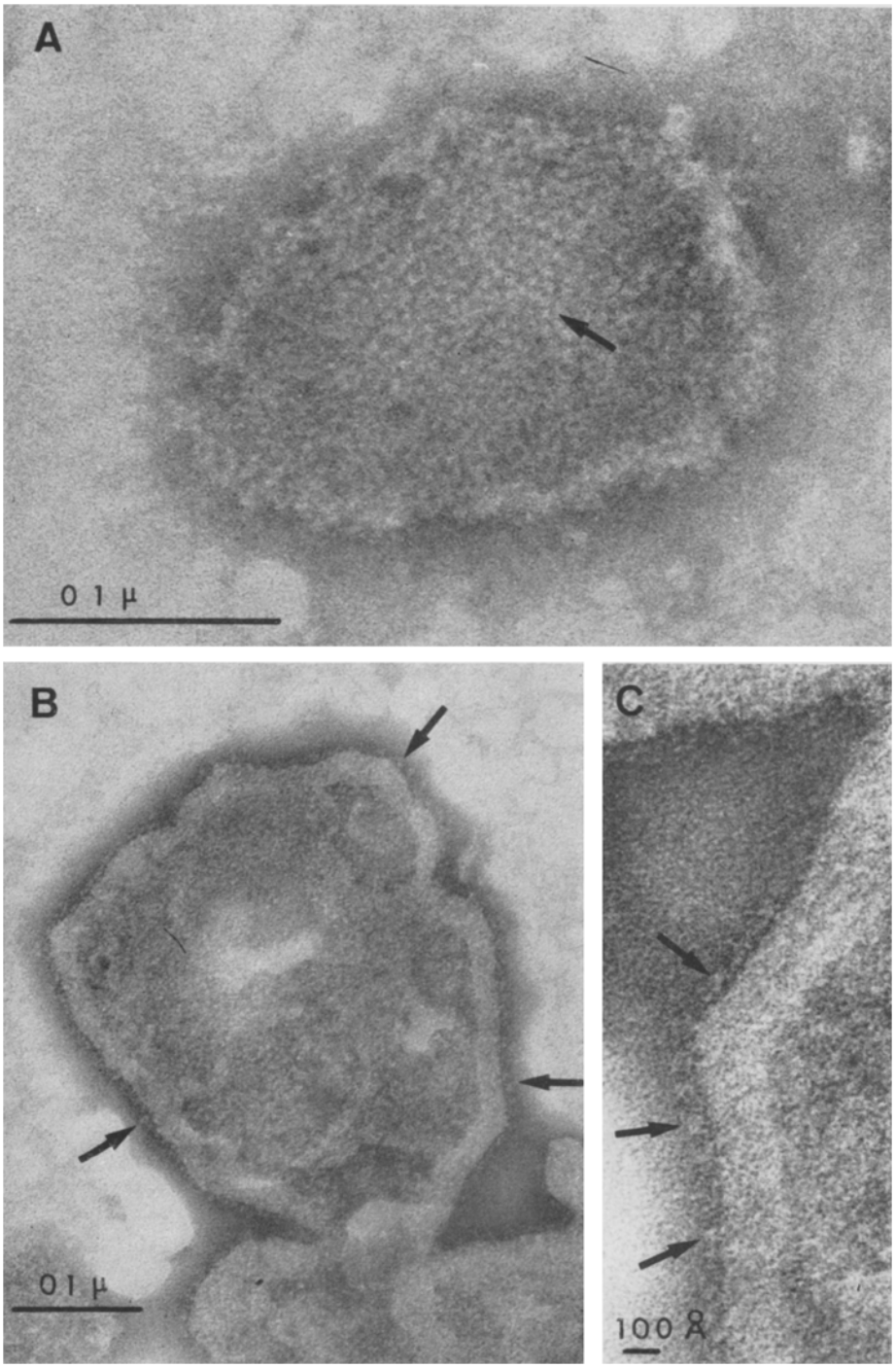

Fig. 2. Negative staining. (A) Microsacs of the AcChE-rich fraction negatively stained by sodium phosphotungstate. The membrane sheets consist of globular subunits with a center-to-center distance of 60 Å. The arrow points to a regular array of subunits. (B) and (C) Microsacs of the ATPase-rich fraction. The membrane surface is covered by globular knobs which are protruding at the edge of the membrane sheets (arrows)

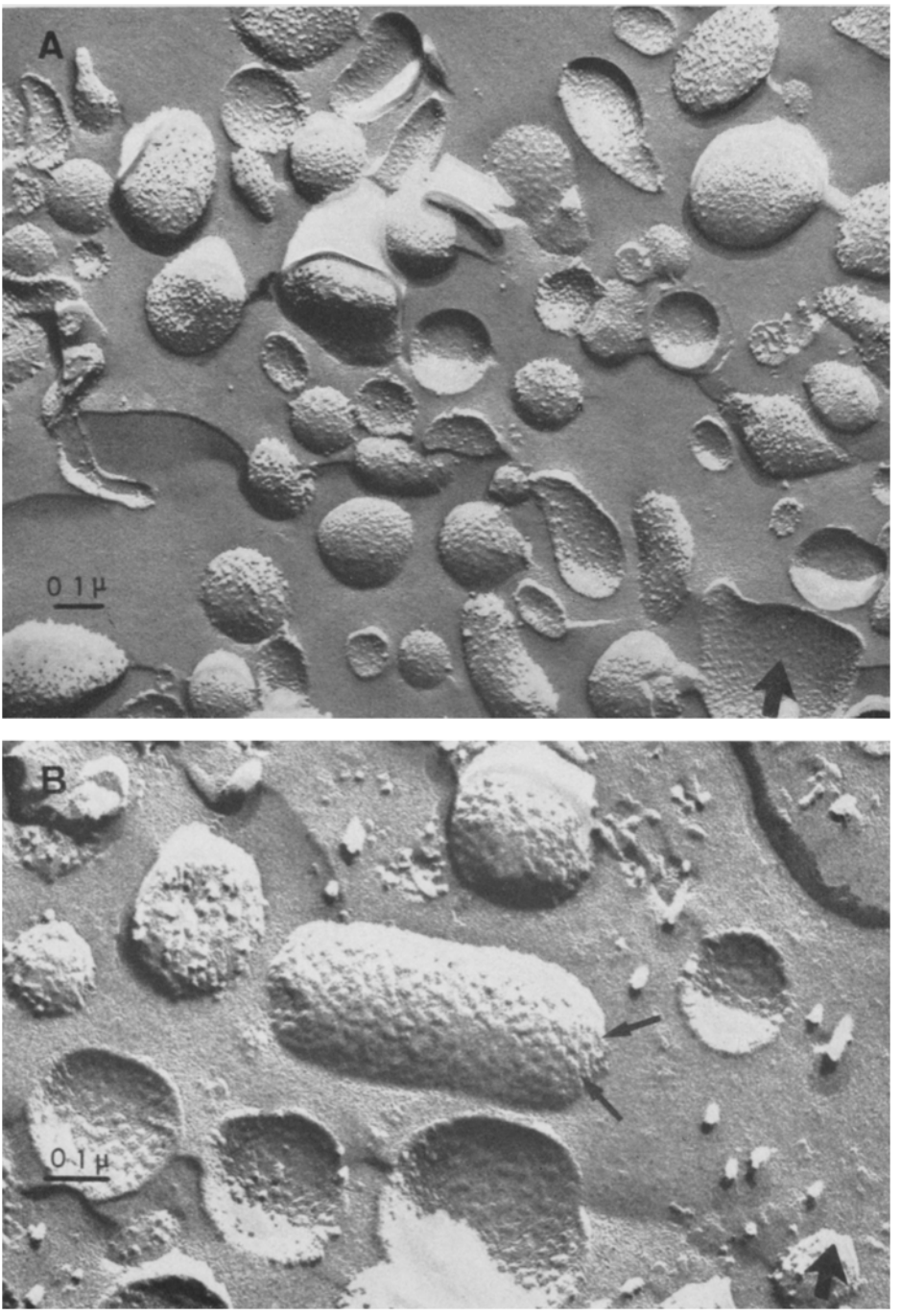

Fig. 3. Freeze-etching. (A) Replica of freeze-etched AcChE-rich microsacs. The picture shows a rather regular size and shape of the fractured microsacs. The exposed surface shows two classes of particles, one forming the frame of the surface, the other protruding from the latter. In all pictures of the replicas, the large arrow indicates the direction of the shadow. (B) Higher magnification of the same preparation, showing an array of repeating units (arrows)

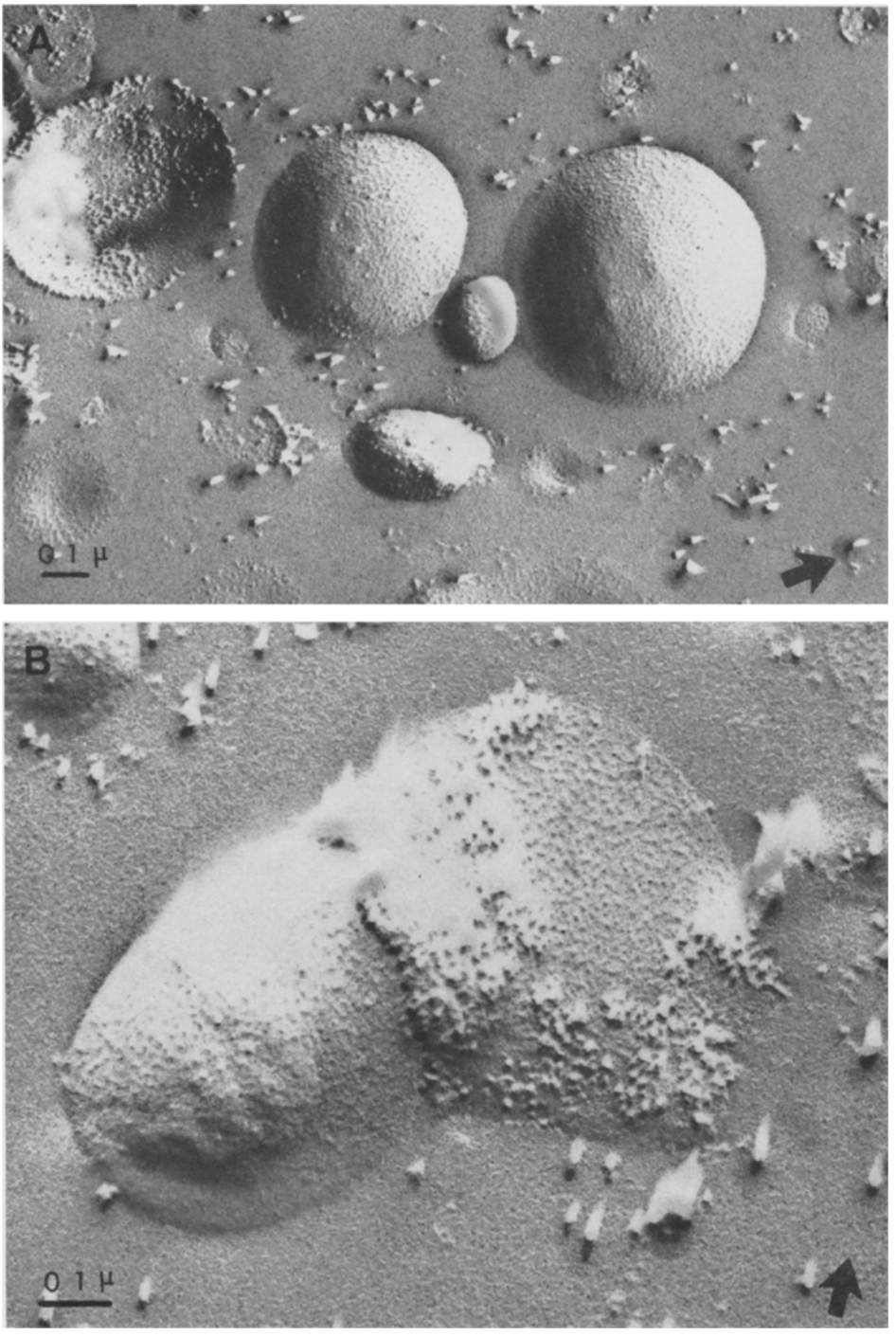

Fig. 4. Freeze-etching. (A) Replica of freeze-etched ATPase-rich microsacs showing a great variety of size of the fractured microsacs. (B) Higher magnification showing that the fractured surface is characterized by small subunits forming the surface frame and by irregularly clustered larger subunits protruding from the surface

However, the distribution, the size and the order of these globules are different depending on the type of membrane considered.

In negatively stained preparations, the globules appear to be integrated *within* the membrane of the AcChE-rich microsacs. In contrast, they make knobs budding *outside* the membrane of the ATPase-rich microsacs. Similar knobs have already been seen with liver plasma membranes (Benedetti & Emmelot, 1965) or inner mitochondrial membranes (Racker, 1969). It is worth mentioning in this respect that, in this last case, the knobs contain a membrane-bound ATPase (Branton, 1969).

By freeze-etching of the microsacs, two types of globular subunits have been observed. The answer to the question of whether these subunits are localized within the membrane core or at the membrane surface depends on the interpretation of the fracture mechanism of biological membranes. Branton has repeatedly given experimental evidence to support the hypothesis that the fracture splits the membranes in two halves exposing the inner membrane surfaces. Therefore the structure *visualized in* or *on* the fractured faces would be *localized inside* the membrane.

The chemical composition of the membrane might also influence its mechanism of fracture. In the case of the electroplax membrane, no definitive conclusion can be proposed about the localization of the repeating units; the analysis of the mechanism of fracture in this particular case is presently under study with both the isolated microsacs and the electroplax membrane *in situ*.

In conclusion, it is clear that the plasma membrane of the electroplax presents a complex organization and that this organization is different on each of its two faces. It is expected that, in the near future, we shall be able to propose a functional interpretation of the observed structure.

We thank Michel Recouvreur, Philippe Breton and Monique Huchet for their expert technical assistance. This investigation was supported by the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, the Fondation pour la Recherche Médicale Française, the Collège de France, the Commissariat à l'Energie Atomique, and the National Institutes of Health.

References

Benedetti, E. L., Emmelot, P. 1965. Electron microscopic observations on negatively stained plasma membranes isolated from rat liver. *J. Cell Biol.* **26**:299.

Branton, D. 1969. Membrane structure. *Annu. Rev. Plant Physiol.* **20**:209.

Racker, E. 1969. *In*: Membrane Proteins, Proceedings of a Symposium sponsored by New York Heart Association, p. 98. Little, Brown & Co., Boston.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.