ELECTRON MICROSCOPIC OBSERVATIONS ON NEGATIVELY STAINED PLASMA MEMBRANES ISOLATED FROM RAT LIVER

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The Davson-Danielli model of membrane structure features a bimolecular leaflet of phospholipids in lamellar arrangement, covered on both sides by protein (1). This model is in keeping with many electron microscopic and other observations on cellular membranes (2–4) and mixtures of phospholipids and proteins (5–8). An alternative model of membrane structure involving a micellar arrangement of the constituents in the plane of the membrane has also been considered, mainly on the basis of observations on lipid emulsions in water (8–12). Evidence for the presence of this type of structure in cellular membranes is scarce. A hexagonal array of subunit facets is suggested by the observations of Robertson (3, 13) on the synaptic disc of the Mauthner cell in goldfish brain, and of Murray (14) on the inner envelope layer of a *Micrococcus* species. Globular micelles in the membranes of the endoplasmic reticulum and mitochondria and in the receptor of frog retina have been described by Sjöstrand (15, 16) and Nilsson (17), respectively (compare for the latter case reference 3, Fig. 71).

That the structure of a membrane may be further differentiated is shown by the regular array of units ("elementary particles") each composed of a globular head (80 to 100 A in diameter), stalk, and base, on the cristae and inner membrane of mitochondria (18). It has been proposed (18, 19) that all membrane systems may contain analogous particles.

MATERIALS AND METHODS

Plasma membranes were isolated from rat liver as described previously (20, 21). The method consisted of homogenization of the liver in dilute bicarbonate at pH 7.5, various centrifugation runs at low speed, and flotation in a sucrose gradient. Samples were incubated for 1 hour at 37°C either in medium consisting of 0.15 M sodium acetate, 0.15 M NaCl, and 0.9 mM $CaCl_2$, at pH 5.5, or in physiological saline or twice distilled water. After incubation the membranes were centrifuged down and washed once with fresh medium, then washed again with twice distilled water at 4°C.

Untreated and pretreated membranes were spread on a cool liquid surface kept at about 2°C (potassium or sodium phosphotungstate (PTA), 2 per cent, pH 7.2), with a clean needle. In other experiments the negative staining (including the drying) was carried out at 37°C. The surface film of spread plasma membranes was picked up on a thin carbon film. The micrographs were taken in a Philips EM 200 microscope, at the instrumental magnification of 41,000 or 71,000, operating at 80 kv. The double condenser system was used routinely with an average spot size of 15 μ; in order to prevent contamination, a specimen cooling device was also used.

RESULTS

Plasma membranes, freshly isolated from rat liver, were kept untreated at 0°C or pretreated, as described above, at 37°C for 1 hour, and then negatively stained with PTA at low temperature. After PTA staining the membranes appeared as large collapsed and distended sacs embedded in an amorphous film of PTA. Areas in which the spread membrane layers were sufficiently thin for high resolution electron microscopy were studied. Some membranes showed a very fine granular structure in surface view and had smooth edges. In other places the edges of the membranes were covered by an orderly array of small globular units with an average diameter of 50 to 60 A (Fig. 1). These units were attached to the membrane layer either directly or by a short constriction about 20 A long; sometimes a small gap less than 20 A wide was observed between the globular unit and the membrane. Globular units of similar diameter were also scattered on the membrane surface in a somewhat disorderly fashion, or lay free (detached) from the membranes in the PTA film.

In each of the pretreated preparations, regardless of the treatment used, a hexagonal array of subunit facets forming the membrane layer was occasionally observed (see Fig. 2). By contrast, this pattern was never observed in untreated membranes. Since it had been shown (8, 9) that the micellar arrangement of lipids in a hexagonal pattern is stable only at elevated temperature, the

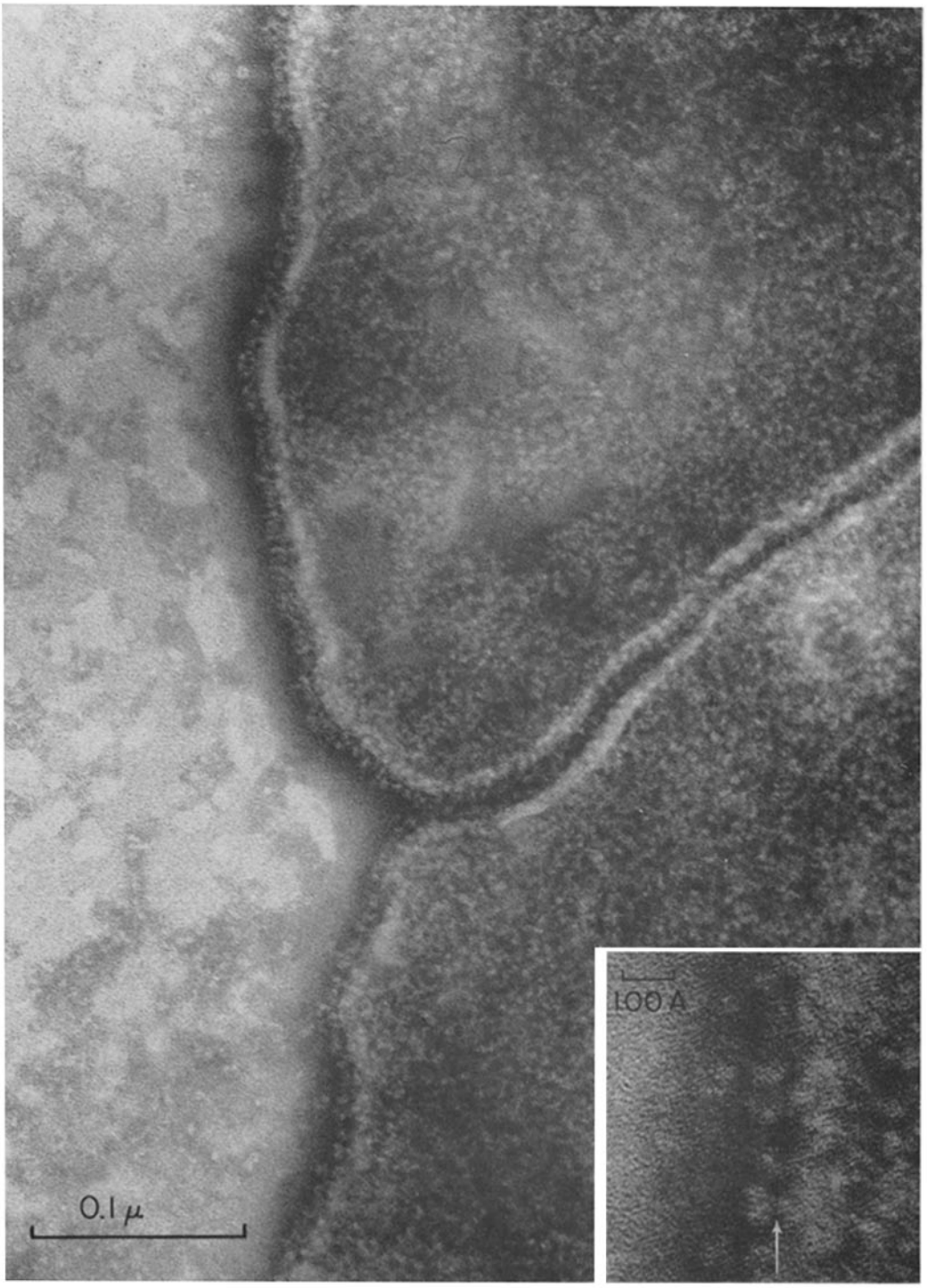

FIGURE 1 Liver plasma membranes negatively stained at 2°C with phosphotungstate immediately after isolation. The surface and the edge of the membranous layers are dotted with small particles having an average diameter of 50 to 60 A. × 320,000.

In the inset the particles are attached either directly or by stalks (arrow) to the outer layer of the membrane. × 720,000.

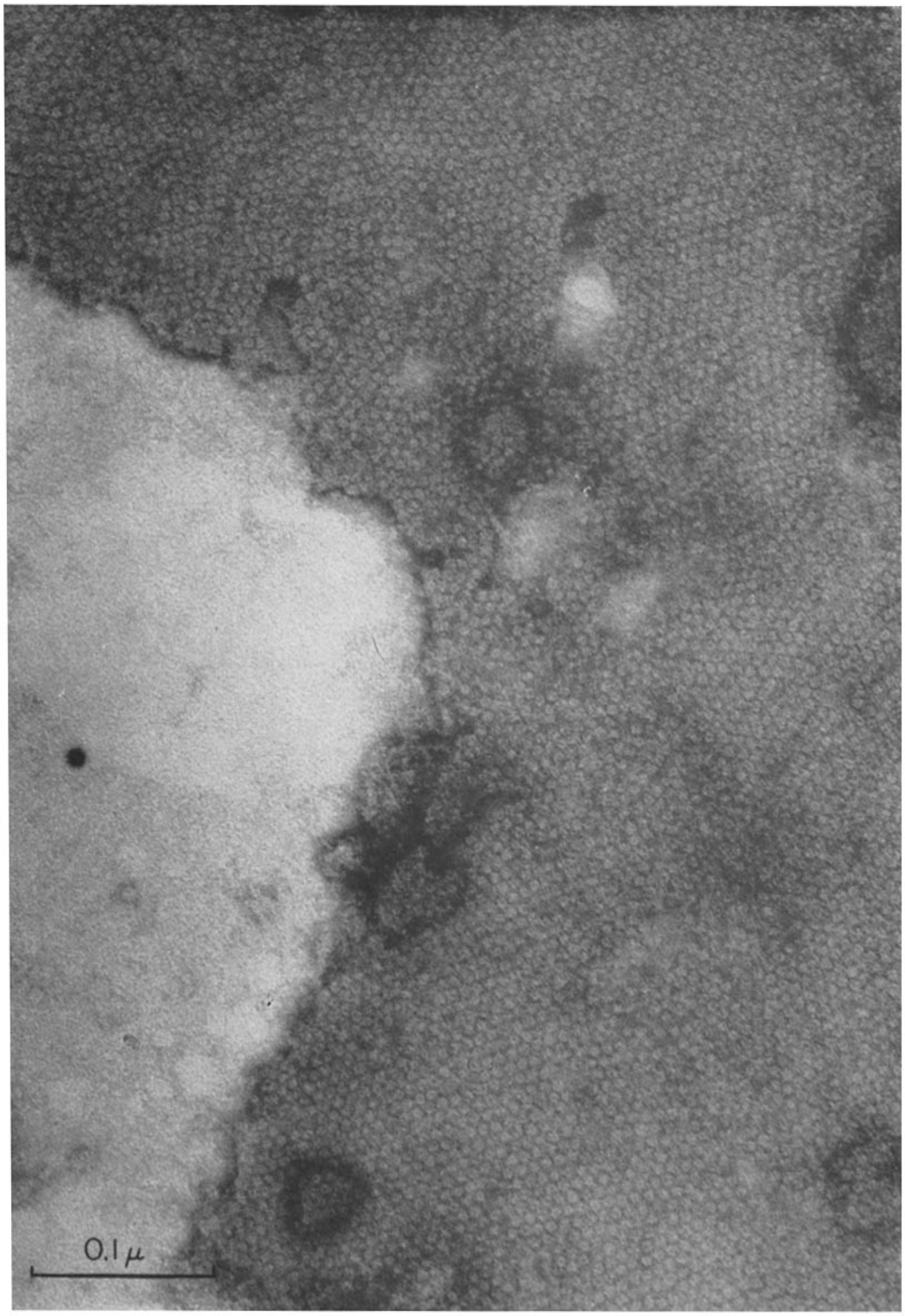

FIGURE 2 Liver plasma membranes negatively stained at 37°C immediately after isolation, showing the hexagonal pattern consisting of a mosaic of packed polygonal facets with a center-to-center distance of about 90 A. × 270,000.

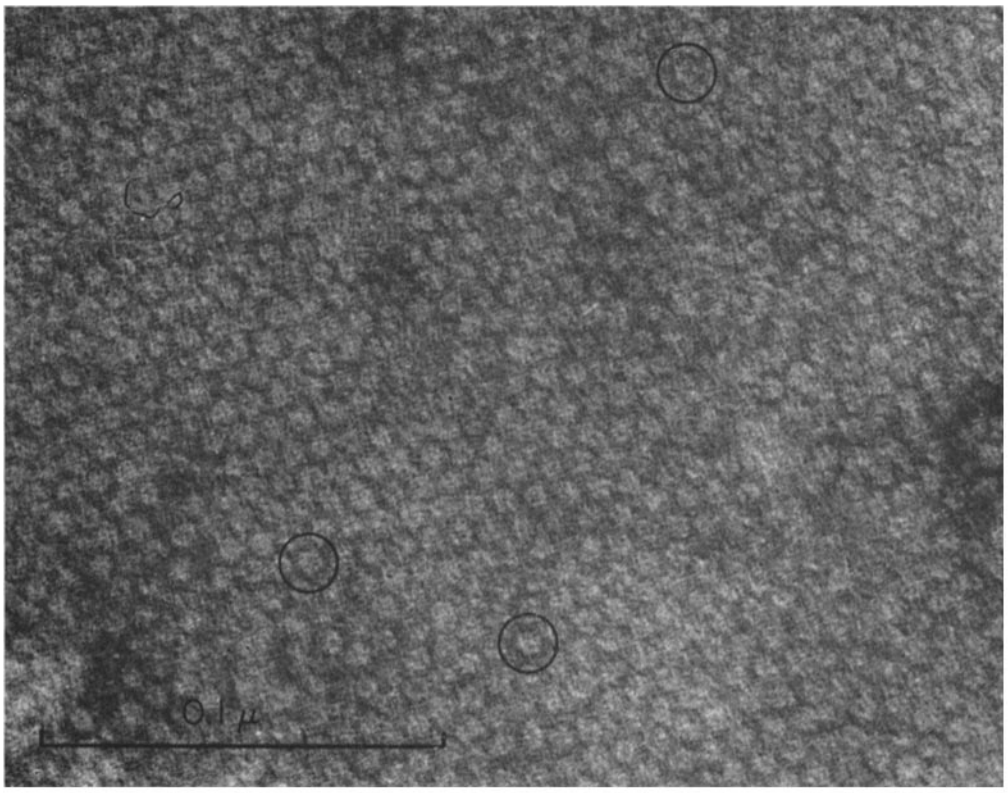

FIGURE 3 High magnification of a portion of the isolated plasma membranes negatively stained at 37°C, showing the hexagonal lattice in which some pentagonal units (circles) are inserted. × 520,000.

possibility was considered that the occasional emergence of the hexagonal pattern after pretreatment of the membranes could be due to the temperature (37°C), this being the only common factor during pretreatment. In order to test this hypothesis, fresh untreated membranes were negatively stained with PTA at 37°C instead of at 2°. Many of the membranes of these preparations now showed the hexagonal pattern over large areas of their surface (Figs. 2 and 3). The hexagonal facets were closely packed, with a center-to-center distance of 80 to 90 A, and were sometimes interspersed with pentagons (Fig. 3). The subunit facets showed a central pit occupied by PTA. Fig. 4 shows that the hexagonal lattice is much enhanced by means of the rotation technique ($n = 6$) (27). Other membranes were covered by the globular units described above, no evidence being obtained that the incidence of these knobs was enhanced as compared with the former conditions. In these preparations, as in pretreated ones, the globular units and the hexagonal pattern appeared to be mutually exclusive, but the two types of structure could be observed in close proximity on separate areas of a membrane sheet.

DISCUSSION

The size and mode of attachment of the globular units of the plasma membranes differed markedly from those of the mitochondrial elementary particles first described by Fernández-Morán (18, 19). These differences have been confirmed directly by us after examination of plasma membrane preparations intentionally contaminated by mitochondria (20). Recently, Sjöstrand *et al.* (22) arrived at the conclusion that the elementary particles are induced artificially as a result of the *in vitro* conditions (water, 37°C) to which the mitochondria are subjected. A rearrangement of the plasma membrane constituents during *in vitro* manipulations cannot be excluded *a priori*. However, the appearance of globular units on the plasma membranes

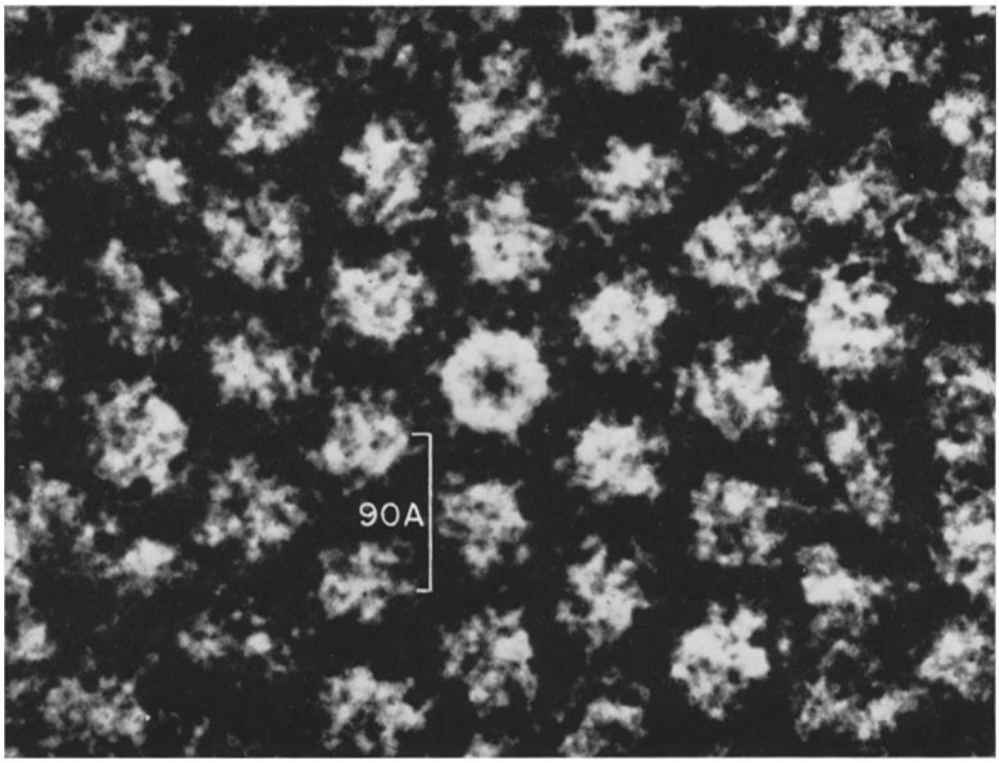

FIGURE 4 Isolated plasma membranes negatively stained at 37°C. The original micrograph has been rotated (n = 6) according to the method of Markham *et al.* (27). The hexagonal pattern and the three-fold axes are enhanced.

was not enhanced after pretreatment with water at 37°C or staining at 37°C, as compared with fresh membranes stained at low temperature. Even if the particles on the cristae and plasma membranes are artificially induced, the finding still remains that different types of particles are formed, pointing to intrinsic structural differences between the two types of membrane, which differ also in enzyme content (20, 21). The globular units are restricted to certain membranes or parts thereof. Although this may mean that the units are easily lost, it may also be that they are confined to specialized regions of the plasma membranes, such as those lining the bile canaliculi, in which certain enzymes, *e.g.* adenosine triphosphatase, are concentrated (23). (Comparable with this would be the local structural specialization of the mitochondrial outer membrane described by Parsons (24).) The globular units resemble the peripheral knobs of the avian myeloblastosis virus (25), which derives its envelope from the plasma membrane. No evidence was obtained in preliminary experiments that this type of globular unit was present on membranes of the smooth and rough endoplasmic reticulum of the liver.

The observation that plasma membrane areas which were covered by globular units did not exhibit the hexagonal pattern might mean that the emergence of the latter structure is prevented or masked by the globular units. The micellar arrangement of hexagonal facets was absent in untreated membranes stained at 2°C, occasionally present in membranes pretreated at 37° and stained at 2°C, and very abundantly present in untreated or pretreated membranes stained at 37°C. These findings strongly suggest that the hexagonal pattern is temperature dependent. The interpretation of this phenomenon might be comparable to that based on the electron microscopic observations of Stoeckenius (7, 8) on lipid-water emulsions. These systems may exist either in a lamellar arrangement or in a micellar one forming

a hexagonal pattern; the latter arrangement is stable only at 37°C, and below this temperature transition of the lipid phase from the micellar to the lamellar arrangement occurs. However, apart from the correspondence between our results on negatively stained plasma membranes and those of Stoeckenius on positively stained fatty material as regards the appearance of a temperature-dependent hexagonal pattern, any further conclusions seem to be unwarranted. For example, in contrast to Stoeckenius' results, it cannot be deduced from our data how the polar end-groups of the phospholipids are oriented. The period of the hexagonal pattern shown by the plasma membranes is about twice that seen in the artificial lipid emulsions (8). Although this difference might conceivably be due to the presence of protein and the size of the phospholipids in the membranes, as suggested by the variations observed (6–8) in the period of myelin figures in model systems according to the choice of lipid and the amount of protein adsorbed, the relevance of these factors for the micellar dimensions remain to be established.

Phospholipid micelles interspersed with proteins feature in the membrane structures proposed by Sjöstrand (15, 16) and Lucy (12). However, the manner in which phospholipid and protein contribute to the hexagonal structure visualized in the plasma membranes is, in fact, not established. Thus, it cannot be decided whether the emergence of the hexagonal pattern is due to a temperature-dependent phase transition of the phospholipids—although this remains an attractive working hypothesis—or to a primary effect on the protein or cholesterol component of the plasma membranes. The period of the hexagonal pattern of the plasma membranes was similar to that of the positively stained synaptic disc membranes reported by Robertson (13). The latter structures also show the central pits in the subunits, among which occasional pentagons were present. The negatively stained bacterial cell membranes described by Murray (14) also showed central pits in the subunits, whose dimensions are larger than those observed in the isolated liver plasma membranes, negatively stained. This difference in the dimensions of the subunits may be due to the particular composition of the two membrane types. Finally, it may appear that in all these cases the subunits possess an icosahedral symmetry and, according to their relative orientation in the plane of the membrane, show up as either hexagons or pentagons. This may provide the necessary flexibility for the membrane sheet (compare reference 26).

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