HEXAGONAL ARRAY OF SUBUNITS IN TIGHT JUNCTIONS SEPARATED FROM ISOLATED RAT LIVER PLASMA MEMBRANES

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ABSTRACT

Solubilization of isolated rat liver plasma membranes in 1% deoxycholate and centrifugation yielded a fraction (pellet) that consisted mainly of tight junctions (zonulae occludentes). An hexagonal array of subunits similar to that previously found in a number of the unfractionated plasma membranes was demonstrated in all the membrane sheets of these preparations by negative staining. It is concluded that the hexagonal subunit pattern is present in the tight junctions, and that this structural differentiation may be related to the intercellular diffusion afforded by the junctional membrane.

INTRODUCTION

In a previous communication (1), we have described the presence of an array of hexagons interspaced with an occasional pentagon in negatively stained plasma membranes isolated from rat liver. The hexagonal lattice was restricted to certain membrane sheets, or parts thereof, suggesting the possibility that the pattern was confined to some specialized regions of the membranes. Recently, Revel and Karnovsky (16) studied thin sections prepared from lanthanum-stained blocks of mouse liver and heart, and observed cell junctions consisting of the plasma membrane leaflets separated by a minute gap, which could be filled by the stain. In these junctional areas, hexagonally packed structures were delineated by lanthanum. This pattern, observed in thin sections, closely resembled the one demonstrated by us after negative staining of isolated liver plasma membranes.

The present paper describes a method whereby tight junctions can be separated as a subfraction from isolated rat liver plasma membranes. Electron microscopic observations are also presented showing that the above-mentioned hexagonal array is a specific feature of tight junctions rather than of the entire plasma membrane.

MATERIALS AND METHODS

Isolation of "Tight Junctions"

Plasma membranes were isolated from rat livers (strains R-Amsterdam or Sprague-Dawley) as previously described (6). The isolated membranes were washed and suspended in 1 mM $NaHCO_3$, pH 7.5, at 2.0–3.0 mg of membrane protein per ml. An equal volume of 2% sodium deoxycholate (DOC) in 1 mM $NaHCO_3$ was added to give a final concentration of 1% of detergent (pH 8.0 in the absence of plasma membranes); the mixture was allowed to stand for 15 min. After centrifugation for 2 hr at 105,000 *g*, a pellet was obtained which was dialyzed for 24 hr against ammonium formate buffer, pH 9.0. All operations were performed in the cold (0°–4°C).

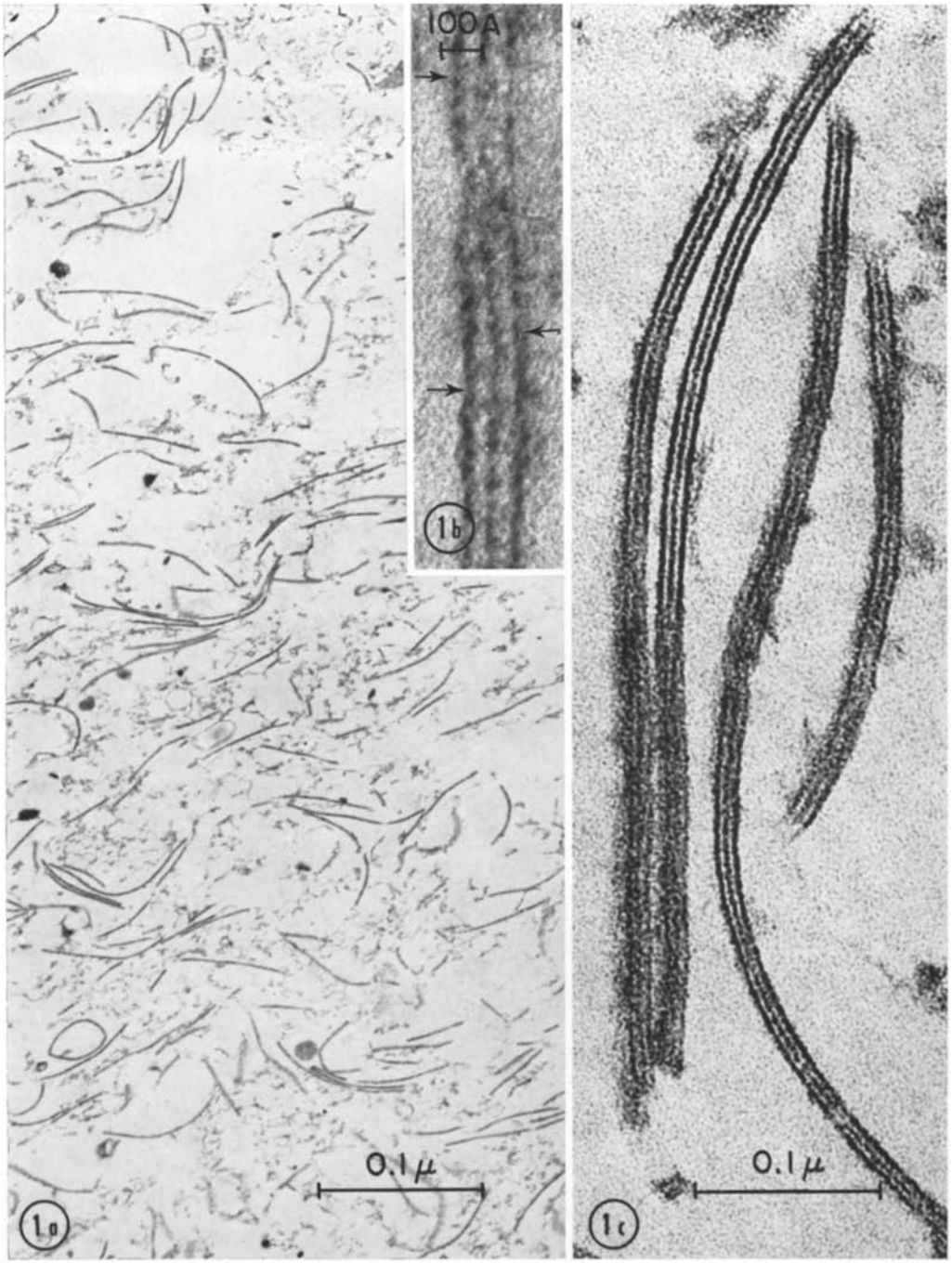

FIGURE 1 *a* Low magnification micrograph (× 23,000) of uranyl acetate and lead-stained section through a pellet from isolated plasma membranes treated with 1% deoxycholate and centrifuged at 105,000 × *g* for 1 hr. The section contains mainly membrane segments and some amorphous material. 1 *c* The membrane segments appear as a pentalayered structure. × 250,000. 1 *b* Higher magnification of a membrane segment in which the pentalayered structure appears to be made up of two rows of globules (arrows). × 600,000. The scale mark on Fig. 1 *a* should be 1 μ.

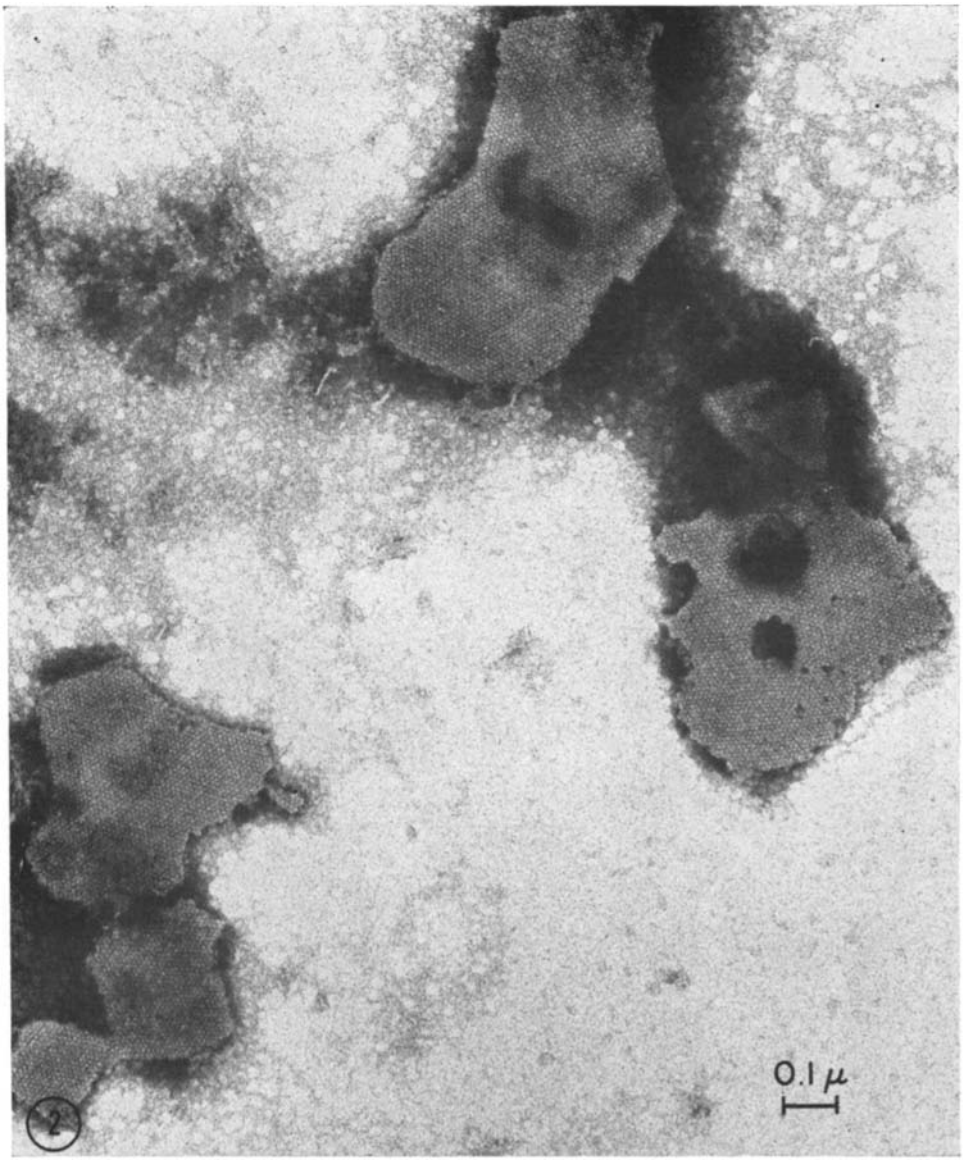

FIGURE 2 Pellet from a preparation identical to that shown in Fig. 1, but negatively stained with phosphotungstate at 37°C. All membrane sheets (tight junctions) show a hexagonal pattern. × 75,000.

Electron Microscopy

The pellet was fixed in phosphate-buffered glutaraldehyde and osmium tetroxide, and embedded in Vestopal. Thin sections were stained with uranyl acetate and lead hydroxide. Phosphotungstate (PTA) was applied as negative stain at either 4° or 37°C, as previously described (1). Large amounts of isolated membranes or of DOC-prepared tight junctions were also spread on thick collodion films and negatively stained with phosphotungstate. The preparations were then rapidly dried at 37°C, and the films stripped from the glass, fixed in buffered glutaraldehyde, postfixed in osmium tetroxide, and embedded in Vestopal. Thin sections of this material were studied either unstained or after being stained with uranyl acetate. All the electron microscopic observations were made with a Philips EM 200 (1).

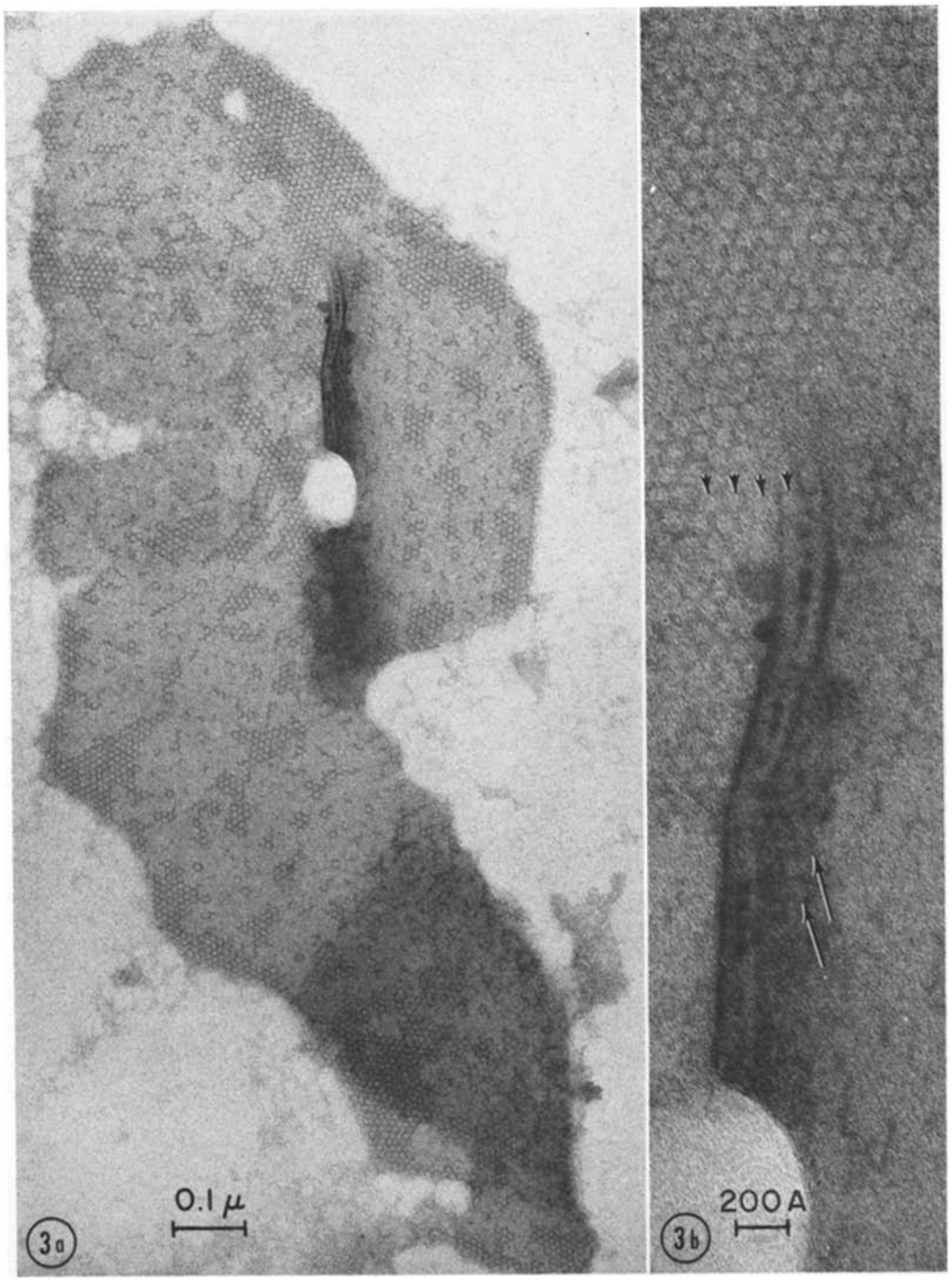

FIGURE 3 Same preparation as in Fig. 2, but the negative stain has been applied at 4°C. *a*, The hexagonal subunit pattern is restricted to certain areas whereas the remainder of the membrane sheet exhibits a homogeneous structure. × 110,000. *b*, In the central region of the sheet a pentalayered structure is present, which is shown at high magnification, × 350,000. The pentalayered structure appears to be made up of two rows of globules, outlined by the stain, which are in series with the hexagonal facets (arrows and arrowheads).

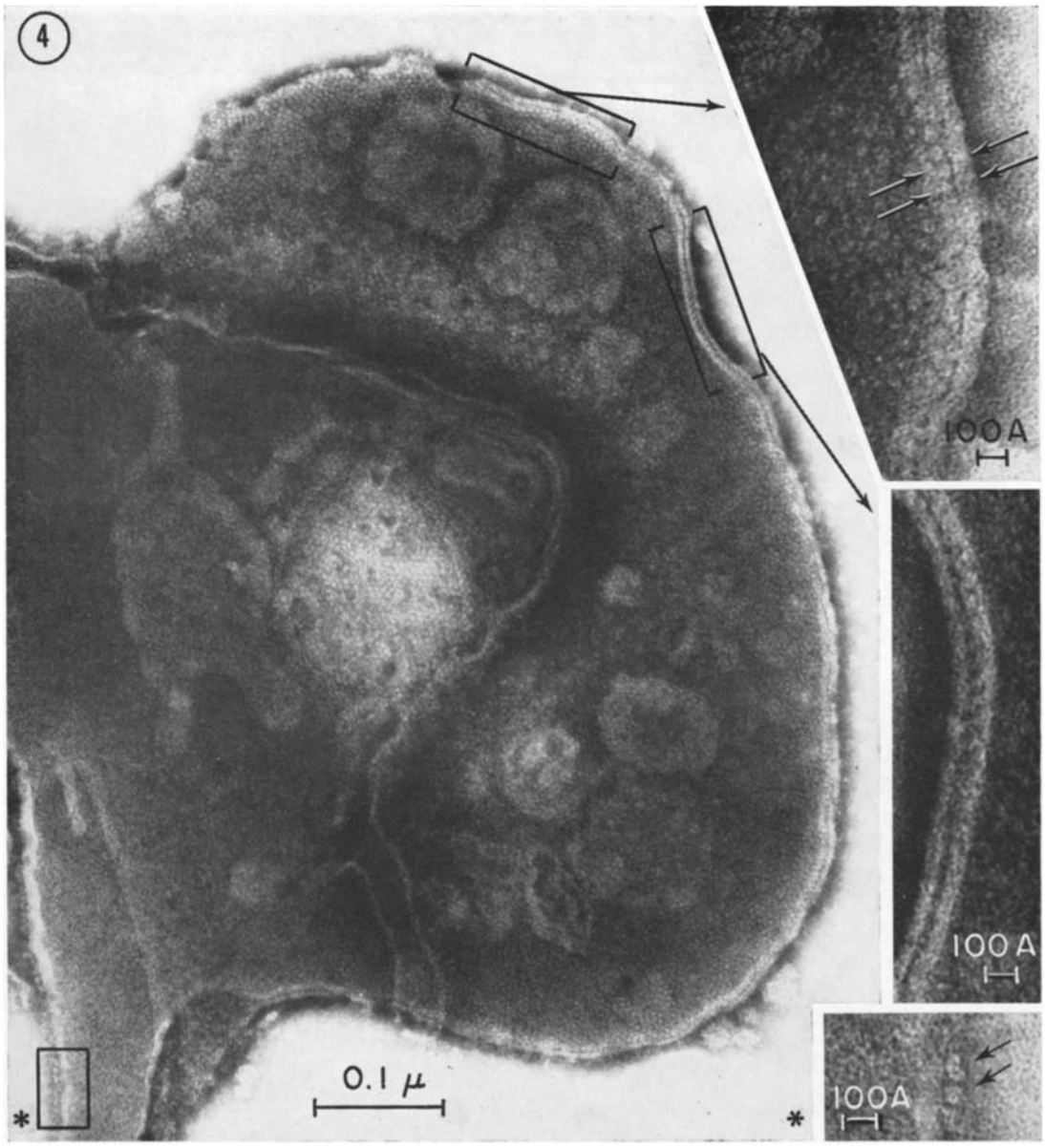

FIGURE 4 Untreated, isolated rat liver plasma membranes which have been negatively stained at 37°C. The large membrane sheet shown in the main micrograph (× 120,000) appears to consist of two layers. A large area of the sheet shows the hexagonal subunit pattern, whereas a region on the left side of the micrograph appears homogeneous, and at one edge (extreme left) globular knobs of the type described previously (reference 2) are visible at the surface. These knobs are also shown at high magnification in the bottom insert (× 400,000). In the other two inserts, two regions of the edge of the membrane sheet are shown at higher magnification. The edge appears to double and to consist of two rows of subunits in series with the hexagonal facets (arrows). × 400,000.

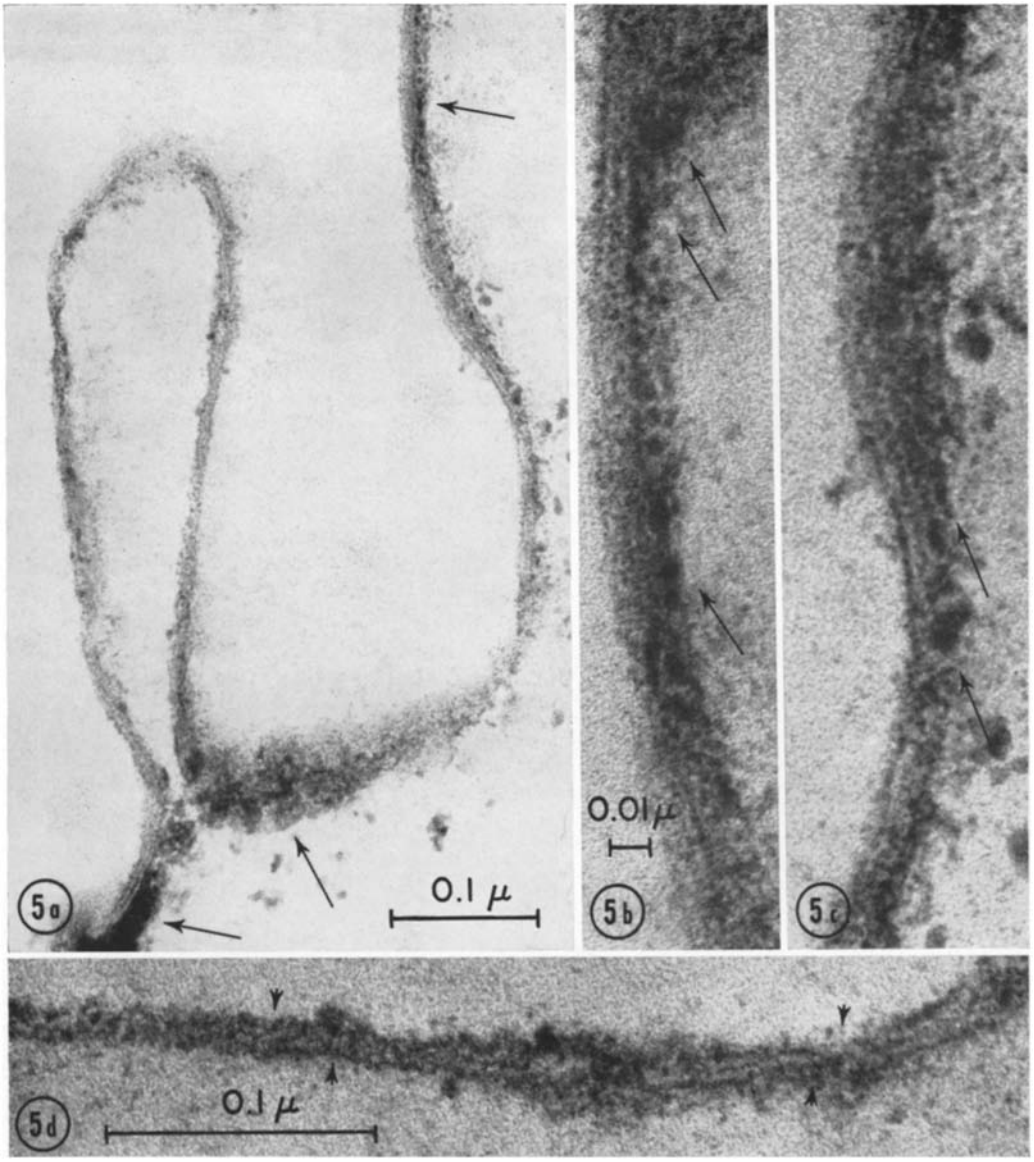

FIGURE 5 Isolated tight junctions negatively stained, fixed, and embedded. The pentalayered structure is preserved, although a little deteriorated. The electron-opaque material (PTA) is accumulated at one side (arrows) of the junction. The arrowheads point to segments of the pentalayered structure in which fragmentation and collapse are evident. Note also the irregular width of the junction. *a*, × 180,000; *b* and *c*, × 500,000; *d*, 350,000.

RESULTS

Examination of thin sections of the pellet, prepared as described under Materials and Methods, showed the presence of many short membrane segments and some flocculent material (Fig. 1 *a*). At high magnification, as shown in Fig. 1 *c*, the membrane segments appeared to consist of a pentalayered structure of about 140 A, having morphological features very similar to those of tight junctions as seen in situ (7), or in isolated liver plasma membranes (2). Frequently, the

intermediate or fusion line of the isolated tight junctions appeared interrupted. Cross-bridges were also visible, at rather regular spacing, between the intermediate line and the inner leaflets (Fig. 1 *b*). In these regions, the tight junctions seemed to be made up of two rows of globules, each about 70 A in diameter.

The isolated tight junctions were also examined after negative staining with phosphotungstate at 4° and 37°C. These preparations (Fig. 2) consisted almost exclusively of membrane sheets, each exhibiting an hexagonal array of hexamers and a few pentamers. The center-to-center distance of the subunits amounted to about 80 A, as previously described for intact membranes (1); the central pit of the subunits was also visible. When the negative staining was performed at 37°C, the isolated tight junctions exhibited the hexagonal subunit pattern over their entire surface (Fig. 2). However, when the negative staining was applied at 4°C, hexagonal arrays were seen interspaced with, or surrounded by, areas in which the membrane sheet appeared homogeneous (Fig. 3 *a*). In some of the membrane sheets (Fig. 3), tears or lines of fracture appeared which showed a pentalayered structure made up of two rows of globular subunits. These globules were "in series" with the subunits of the hexagonal array of the membrane sheet (Fig. 3 *b*, arrowheads). Globular subunits were visible also in what seemed to be the side (slope) of the pentalayered structure (Fig. 3 *b*, arrows). A double edge was also seen in hexagonal areas of isolated rat liver membranes, untreated with DOC and negatively stained at 37°C (see Fig. 4).

In thin sections of negatively stained and embedded preparations, tight junctions were still recognizeable even though frequently fragmented and twisted (Fig. 5 *a*, *d*). Many of the junctions had a variable width from about 100 to 130 A. In these preparations, tight junctions, when cut normally to the plane of the membranes, consisted of two sheets of low electron opacity, each about 50 A thick, separated by an electron-opaque "intermediate" line and outlined on each side more by an accumulation of an electron-opaque material, of variable thickness, than by two distinct uniform layers (Fig. 5 *a–c*). The electron-opaque material, probably phosphotungstate, was more abundant on one side of the junctions, most likely that corresponding to the side next to the collodion film. The "intermediate" line was frequently interrupted and here and there appeared very thin (Fig. 5 *d*). In the preparations so far studied, no cleavage of the junctions into two separate membrane sheets was ever observed. In oblique sections the junctions were characterized by a local striated pattern, tenuously resembling the hexagonal array seen in identical preparations negatively stained but not embedded (Fig. 5 *b*, *c*).

DISCUSSION

Isolated rat liver plasma membranes consist of large sheets interconnected by junctional complexes identical to those present in situ. According to Farquhar and Palade (7, 8), three types of junctions can be distinguished in epithelial cells. Of these, the tight junction, serving as a "closing belt" (8), is the most resistant to mechanical tension (7, 8), and to withdrawal of divalent cations by ethylenediamine tetra-acetate (2, 3, 22). The present investigation shows that the tight junction of rat liver plasma membranes is also resistant to the lytic effect of deoxycholate. Thus, solubilization in DOC can be exploited for separation of tight junctions from the remainder of the plasma membrane.

The reason for the remarkable resistance of tight junctions, in comparison with the rest of the plasma membranes, to solubilization by DOC is not readily apparent. It seems likely, however, that this resistance is dependent upon changes in the configuration of the plasma membrane occurring during formation of the tight junction which may or may not be mediated by the elimination and/or the addition of some constituent(s). The fact that sialic acid could be demonstrated in isolated rat liver plasma membranes, as well as in desmosomes and intermediate junctions, but not in tight junctions is consistent with this view (2). Moreover, Robertson (18) and Revel and Karnovsky (16) demonstrated that the membrane element of a similar type of intercellular junction has a characteristic structural pattern, the hexagonal array of subunits, which is different from that of any other portion of the plasma membrane. Our present observations confirm these findings and show that the hexagonal pattern revealed in negatively stained preparations is confined to the membranes forming the tight junctions. It is noteworthy also that this pattern could not be demonstrated in isolated plasma membranes of rat hepatoma which are devoid of tight junctions (4).

The appearance of the hexagonal subunit pattern in unfractionated rat liver plasma membranes was previously found to be temperature dependent. This finding was tentatively explained on the basis of the phase transition that phospholipids are known to undergo when the temperature is raised to 37°C and the water content is reduced (14, 23). However, a similar pattern was subsequently found in plasma membrane isolated from mouse liver (3, 4) after negative staining at either high (37°C) or low (4°C) temperature. It seems, therefore, that the condition(s) for the formation or appearance of such a pattern differ substantially in rat and mouse liver, and/or that structural variations of these junctions are present in the two animals.[1]

According to Revel and Karnovsky (16), two types of tight junctions can be distinguished: (*a*) one corresponding to truly "tight" junctions (zonula occludens) with a complete obliteration of the intercellular gap, and (*b*) the "close" type characterized by the presence of a minute gap between the external leaflets. In the latter junction, the penetration of electron-opaque substances outlines the hexagonal pattern. The "close" type has been found in mouse heart and liver (16) and in the smooth muscle of guinea pig colon (17); the tight junction, in proximal kidney tubules and in intestinal epithelium (16, 17). Under the conditions of fixation and staining used in our work, tight junctions were almost exclusively found (2–4) in isolated rat liver plasma membranes. If the hexagonal array of subunits is purported to be a unique feature of "close" junctions, the appearance of an identical pattern in rat liver tight junctions after negative staining at 37°C or after DOC treatment independent of the temperature remains to be explained. One possible explanation is that the "tight" junctions of rat liver are of the "close" type, but our fixation, embedding, and staining procedures were not adequate to show in thin sections the intercellular gap. This possibility, however, seems unlikely since under identical technical circumstances "close" junctions were easily revealed in isolated mouse liver plasma membranes,[2] in agreement with the observations of Revel and Karnovsky (16). A second possibility is that the DOC treatment and the negative staining at 37°C may cause either the solubilization of junction component(s) or a reorientation of the relative positions of part of the lipids and proteins in the membrane. Both of these conditions might facilitate the penetration of the electron-opaque material, thus revealing the hexagonal pattern in the junction. Evidence that negative staining with PTA per se affects the structure of the junction is provided by the findings in thin sections of negatively stained membranes in which reduction in width and fracture and twisting of the pentalayered structure were observed (see Fig. 5). Indications consistent with the induction of structural modifications in this type of junction by the preparative procedure has also been obtained by others (21). Therefore, it is not clear whether the "tight" and "close" junctions are separate entities, or represent structural modulations of the same junctional unit. This problem is important not only from a structural point of view, but also with respect to the function of the junction. The tight junction has been considered to be a region of the cell surface which shows low electrical resistance and where intercellular communication may proceed unimpaired (13, 15). For the insuring of such selective permeability, according to Loewenstein (13), the junctional unit must be insulated from the extracellular environment. In other words, the "surface diffusion barrier" (see Fig. 22 of reference 13) must not be leaky. Lanthanum penetration into the intercellular gap present in the "close" junction implies leakiness across and between the adjacent cells. If this condition is still compatible with high permeability from cell to cell at the junctional membrane, the concept of the junctional unit postulated by Loewenstein (13) should be revised.

Most of the tight junctions in negative contrast appear as single sheets of hexagonal facets. However, when a double edge (see Fig. 4), or a fold or tear in the plane of the membrane (see Fig. 3) is viewed, there is a suggestion that the sheets may consist of two identical layers. The same is true in tight junctions negatively stained and then fixed and embedded. Here, cross-sections

[1] Chemical differences between isolated rat and mouse liver plasma membranes have been observed. For instance, the cholesterol content of plasma membranes isolated from CBA mouse liver is some 30% higher than that of rat liver plasma membranes (Emmelot and Bos, unpublished).

[2] E. L. Benedetti. 1968. The structure of junctional complexes isolated from rat and mouse liver. In preparation.

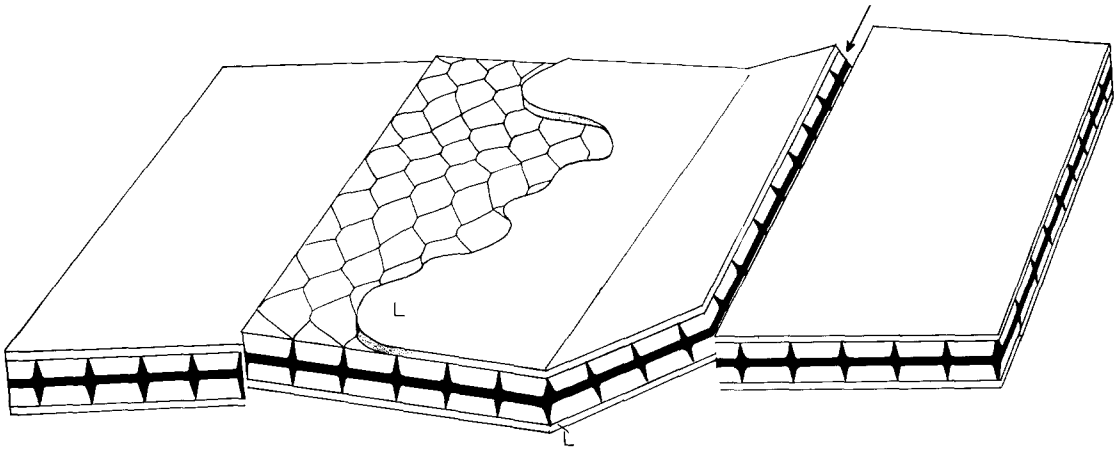

FIGURE 6 Tentative diagrammatic representation of isolated tight junction. The dimensions of the structure depicted are not drawn to scale. The central dark area represents the surface of contact between the outer leaflets of the adjacent membranes. The hexagonal internal pattern of each leaflet is seen in strict register. The hexagonal subunit pattern seen in negatively stained preparations is probably the result of projection of both leaflets. The inner leaflets (*L*), probably being more transparent, do not participate in the image formation. The arrow indicates a tear or fracture in which the junction is visualized in edge view (compare with Figs. 3 and 4). The drawing does not refer to any particular molecular arrangement of the junctional complex; this arrangement, as yet, has not been established.

through the junction show the persistence of two layers, but not cleavage of the junctional membranes. The resulting image of the hexagonal array of subunits may, therefore, arise in this instance from a projection pattern of two superimposed hexagonal lattices (see Fig. 6). Since a "moiré" pattern has very seldom, if ever, been observed, the two leaflets must be tightly bound so that lateral or rotational displacement probably cannot occur (11). Both the "near" and the "far" hexagonal lattice would contribute to the final contrast image in the negatively stained membrane (9, 10, 12) although the side "near" to the supporting film probably dominates the other side, owing to local accumulation of electron-opaque material (see Fig. 5).

The hexagonal array of facets that Robertson described in synaptic discs has been referred to as a differentiation ("granulofibrillar structure") of the outer surface of each of the two membranes forming the disc (19, 20). This surface differentiation would have a limited extent and probably involves protein, but not the lipid layers (19). Also, the observations of Revel and Karnovsky (16) on lanthanum-stained tissue strongly indicate that the hexagonal subunit pattern is a surface feature of both the leaflets where they are "close" joined or merging together.

Another alternative is that the globules visualized in thin sections (see Fig. 1 *b*) and the subunits outlined in the negatively stained preparations (see Fig. 3 *b* and 4) are the same structural entities. In such a case, the macromolecular repeating units forming the hexagonal array could tentatively be considered to consist of a lipid and/or hydrophobic protein core, containing a central hydrophilic hole, and coated by hydrophilic protein or ionogenic groups of lipid. In any case, it seems likely that the amount of protein governs the dimensions of the hexagonal pattern, since treatment of isolated rat liver membranes with trypsin or papain which releases at least 50% of the membrane proteins did not prevent the appearance of the hexagonal array of subunits in negatively stained preparations, but reduced the size of the hexagonal facets (3, 5). Most likely, the hexagonal subunit pattern, however, does not emerge at the cytoplasmic surfaces (inner) of the junction, since the hexagonal pattern present in negatively stained tight junctions is no longer visible when the same preparations are shadowed by carbon-platinum, either fron a single direction, or while rotating the specimen.[2] Under such conditions, the increased electron opacity of the exposed "inner" surface of the junction probably masks the hexagonal pattern situated underneath.

It is hoped that the method described here, whereby tight junctions can be separated from the rest of the plasma membrane, may help to elucidate some of the unresolved questions concerning these specialized structures.

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