THE ISOLATION OF A CELL MEMBRANE FRACTION FROM RAT LIVER

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ABSTRACT

A procedure is described for isolating cell membranes from rat liver homogenates. 20 gm. of rat liver was homogenized in a Dounce homogenizer in ice cold water buffered to pH 7.5 with $NaHCO_3$, rupturing all of the cells and most nuclei. The diluted homogenate was filtered through cheesecloth to remove precipitated nucleoprotein and centrifuged at 1500 *g*, 10 minutes, to sediment a crude membrane fraction. The membrane containing sediment was recentrifuged 3 times in conical tubes (1220 *g*, 10 minutes), the top layer of the 2-layered sediment being retained. Flotation in a sucrose solution d = 1.22 freed the preparation from contaminating cell fragments and nuclear membranes not previously disintegrated. The floating material ~0.4 ml. was quite homogeneous and consisted of thin amorphous membranes. Electron micrographs revealed numerous double profiles similar in shape and dimensions to apposed liver cell membranes in intact tissue.

Although the cell membrane has been the object of numerous and varied morphological investigations (1), and cell surface enzymology has been extensively studied (2), the investigation of structural and functional interrelationships of the cell membrane has been hampered by the difficulty of obtaining relatively pure preparations of cell membranes. Thus, while such problems as active and passive transport, fibrilogenesis, synthesis of soluble proteins (2), and hormone mechanism of action (3) have all been considered in terms of the cell membrane, direct chemical information has been lacking. The cell membrane is a well defined entity in electron microscopical morphology, yet the relationship of this entity to such processes as active and passive transport is not well understood. The present communication describes methods of isolation and identification of a fraction considered to consist of hepatic cell membranes.

The idea that cell membranes are present in tissue homogenates is not a new one. In 1951 Palade made passing reference to the fact that rat liver homogenates contained "cell membranes" consisting of "1 μ thick sheets of amorphous refringent material" (4). A year later Hogeboom and associates noted "a number of irregularly shaped very thin structures" contaminating their rat liver nuclear fraction and thought these structures to be collapsed cell membranes (5). Dounce (personal communication) observed the same structures, thought them to be cell membranes, and noted them concentrated in the fluffy layer above

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the nuclei in his procedure for isolation of rat liver nuclei (6).

Four methods for obtaining cell membranes have been found in the literature. The best known is the isolation of red blood cell ghosts by centrifugation of red cell hemolysates (*cf.* 7). Boell and Nachmansohn found it possible to isolate the myelin sheath, contaminated by connective tissue, of giant squid axon by dissecting the nerve and squeezing out the axoplasm (8). By microdissection of formalin-fixed amoebae the pellicle has been isolated (9). Membranes of mouse ascites tumor cells have recently been isolated by differential centrifugation after sonic disruption, however, adherent cytoplasmic particles contaminate this preparation (10). Our interest in obtaining membranes from cells responsive to certain hormones, together with the remarks of Palade, Hogeboom, and Dounce mentioned above, led us to attempt to isolate cell membranes from rat liver.

EXPERIMENTAL

Procedure for Making Homogenates and Observing Cell Disintegration: Livers were obtained from either male or female Wistar or Sprague-Dawley rats, varying in weight from 100 to 380 gm. Younger rats were more satisfactory than older ones because cellular breakage occurred more readily in young rats. Animals were stunned by a blow on the head, decapitated, and exsanguinated. The liver was immediately excised and any attached muscle as well as a major portion of the connective tissue of the porta hepatis was dissected away. The liver was then placed in a chilled beaker and minced with scissors. 7 gm. of the minced liver was weighed and placed in a Dounce homogenizer (6) surrounded by a refrigerated water bath, 0–2°C. 28 ml. of appropriate chilled medium (0–2°C.) was added, and the tissue homogenized with 3 to 10 vigorous strokes of the loose pestle. The media used were: (*a*) 0.44 M sucrose pH adjusted between 6.0–6.2 with approximately 0.7 cc. 0.1 M citric acid after the method used by Dounce for the isolation of nuclei; (*b*) 2.2 M sucrose; (*c*) 0.88 M sucrose; (*d*) distilled H_2O, pH 5.5; (*e*) distilled H_2O with sufficient $NaHCO_3$ added to raise pH to 7.5. Final concentration of $NaHCO_3$ was approximately 0.001 M.

The homogenates were observed and photographed through a Spencer AO phase contrast microscope using the high dry (× 43) dark objective and × 15 oculars. For detailed observations the homogenates were diluted about 1:50 with the appropriate homogenizing media.

The entire process of cell breakage and membrane separation could be observed in the following manner. One drop of homogenate was placed on a slide and covered with a coverslip. By tapping gently on the coverslip currents were produced which ruptured the cells and dispersed the cellular particles leaving behind the cell membrane. This procedure was particularly successful when one portion of a cell was adherent to either the glass slide or coverslip.

Procedure for Isolating Cell Membranes: Rat livers were obtained as described above. All procedures were carried out in a refrigerated water bath 0–2°C. and refrigerated centrifuges 0–4°C. The listed centrifugal fields were computed for the bottom of the tube. The densities of the sucrose solutions used in the flotation were computed from data in the Handbook of Chemistry and Physics, 32nd. edition, 1950, pp. 1727–1728. The densities listed in this paper refer to the densities of these solutions at 20°C.

5 gm. of minced rat liver were homogenized with 25 to 30 vigorous strokes of the loose pestle in a Dounce homogenizer using 25 ml. H_2O containing 0.001 M $NaHCO_3$, pH 7.5 (adjusted prior to homogenization) as medium. This procedure ruptured virtually all of the cells and ~90 per cent of the nuclei. The homogenate (4 batches) was poured into 500 ml. of the same medium and stirred for 2 minutes. The diluted homogenate was quickly filtered in succession through 4 layers of both No. 90 and No. 120 cheesecloth in order to remove precipitated nuclear protein and connective tissue. The suspension was placed in four 250 ml. bottles and centrifuged at 1500 *g* for 10 minutes. The supernatants were decanted and discarded. 5 ml. of medium was added to each bottle which contained about ½ cm. thickness of jelly-like, light reddish-tan precipitate, and the precipitate was crudely resuspended by stirring vigorously with a glass rod for about 10 seconds. The crude suspension was poured into the homogenizer and the bottles rinsed with 2.5 ml. of medium in order to transfer the remaining precipitate to the homogenizer. Resuspension was completed in the homogenizer with 3 gentle strokes of the pestle. 15 ml. more of medium was added to the homogenate and the total was placed in three 15 ml. conical centrifuge tubes and centrifuged at 1220 *g* for 10 minutes. The centrifuge was decelerated slowly to avoid stirring and resuspension of the poorly packed precipitate. The supernatants, consisting mostly of mitochondria but containing fair numbers of membranes, were drawn off with a pipet attached to water suction and discarded, and 3 ml. of medium was gently layered with a pipet over the precipitates. The precipitate consisted of a 2-layered pellet at the bottom 0.1 ml. of the tube over which lay 3 ml. of loosely packed pale tan material. The lowest layer of the pellet was white and composed of glass fragments plus adherent cytoplasmic particles. The top layer was red and

composed of erythrocytes which had only partially lost their hemoglobin, leukocytes, nuclei, and membranes. The fluffy portion of the precipitate was stirred from the top gradually working down and resuspending it. Care was taken not to resuspend the bottom layer of the pellet. The partially resuspended material from each tube was decanted into the homogenizer and resuspended in 30 ml. of medium with several strokes of the loose pestle and then recentrifuged as before. The precipitates (1 ml. per tube) were again resuspended this time in 10 ml. of medium, care again being taken to leave the bottom layer behind. Centrifugation was repeated as before, except only one tube was used. At this point, the precipitate (about 0.6 ml.) appeared pale grey, almost white. No bottom layer formed. The supernatant was removed as before. It was found advisable at this point to check one drop of the material using the phase contrast microscope. If mitochondria were present, the washing was repeated. A number of swollen nuclei were present as well as a few membranes derived from nuclei and leukocytes. These contaminants were removed by flotation, since they have a density greater than 1.22. The marked reduction in volume of the precipitates between the first and fourth centrifugations, about 95 per cent, is necessary to free the preparation of mitochondria. This is because of the high ratio of mitochondria to membranes in the initial homogenate, the relatively small difference between the sedimentation rates of the two particles, and the poor packing of cell membranes as compared to mitochondria.

The precipitate was resuspended in 3.3 ml. of water containing $NaHCO_3$ by stirring with a glass rod and then by homogenizing in a small Dounce homogenizer with 3 strokes of the loose pestle. 3.1 ml. of the membrane suspension was then pipeted into a lusteroid centrifuge tube. To this 5.8 ml. of a solution containing 69 per cent by weight of sucrose in water (d = 1.34) was added slowly with constant stirring, bringing the density of the suspension to 1.22. Rapid addition of the sucrose solution may cause aggregation of the membranes and contaminants. Over this, a solution containing 37 per cent by weight of sucrose in water (d = 1.16) was carefully layered with a pipet until the tube was filled. The tube was capped and centrifuged at 100,000 *g* for 75 minutes. (A swinging bucket rotor is preferred for this step. If only angle heads are available, the best results will be obtained with the head in which the tubes are most nearly horizontal.) After centrifugation, the floating material was found to be at the interface between the 2 layers and, with angle heads, adhering to the tube wall closest to the axis of rotation. Both the floating and adhering material were removed by inserting a No. 20 needle on a syringe through the cap screw opening and applying gentle suction while directing the needle at the interface, or scraping the beveled edge along the tube wall.

The entire procedure was easily accomplished in 4 hours and yielded between 0.3 and 0.6 ml. loosely packed material. The material removed was homogenized in 8 ml. of medium and a small amount examined for purity using the phase contrast microscope (Fig. 5). The remainder was centrifuged in a conical 15 ml. tube at 2880 *g* for 30 minutes. The supernatant fluid was discarded and the pellet fixed in suspending medium containing 2 per cent osmium tetroxide for 8 hours and embedded in *n*-butyl methacrylate for electron microscopy. It was then sectioned, stained for 30 minutes with lead hydroxide (11), sandwiched with formvar (12), and examined with the electron microscope.

OBSERVATIONS

Examination of Material after Homogenization: The homogenates contained large numbers of individual or clumped whole cells, broken cells, mitochondria, and nuclei. The polyhedral liver cells displayed hexagonal and rectangular surfaces, but when intact, a surrounding membrane could not be resolved (Fig. 1). After rupture and partial extrusion of cytoplasm, a thin line could be resolved at the cell periphery (Figs. 2 and 3). Such peripheral lines were often continuous with lines radiating outward 5 to 15 μ from the apices of the angular cell surfaces. These radiating formations appeared to represent the borders of previously adjacent liver cells, and often fragments of cytoplasm were lodged around them (Fig. 3). By tapping gently on the coverslip, thus rolling over these structures, the radiating formations were seen to be thin, amorphous sheets. As the cells disintegrated, the radiating formations and peripheral lines fragmented and separated from the rest of the cell and became free-floating structures visualized as thin planar sheets joined at their edges. The sheets were poorly visualized except where abruptly changing orientation, or when viewed on edge. In the latter case they displayed angular "Y," "V," and "L" configurations (Fig. 4).

The separation of peripheral, amorphous sheets from nuclei undergoing disintegration was also observed. These structures were flat and free of bends, and were difficult to visualize even when viewed on edge, in contrast to the cell membranes.

The process of separation of peripheral sheets from cells as described was similar in all media tried. The buffered H_2O medium finally selected

FIGURES 1 to 6

Phase contrast photomicrographs × 700. Rat liver homogenates in 2.2 M sucrose. Similar structures are seen in dilute sucrose solutions and water media. The homogenates have been photographed in the more viscous media to minimize motion of the particles.

FIGURE 1

Two intact liver cells adherent to each other. The bounding cell membranes can not be resolved in intact cells. The arrows point to the sheet-like membranes of the previously adjacent liver cells which have been ruptured by homogenization.

FIGURE 2

A ruptured liver cell with a portion of its cytoplasm dispersed. Note the hexagonal configuration of the cell profile. The bounding cell membrane is resolved as a thin dark line. The radiating dark lines from the cell apices are the cell membranes of previously adjacent cells viewed on edge in contrast to the membranes in Fig. 1 which lie parallel to the plane of focus.

FIGURE 3

Two adherent cells both ruptured. The bounding membranes are visualized. The remains of a previously adjacent cell are seen to consist of two membranes viewed on edge with some mitochondria between them (at the arrow).

FIGURE 4

A free-floating cell membrane viewed on edge. The angulated configuration of the liver cell surface seen in Fig. 2 is maintained and imparts a distinguishing characteristic to the membrane. This is referred to in the text as "Y" and "V" configurations.

FIGURE 5

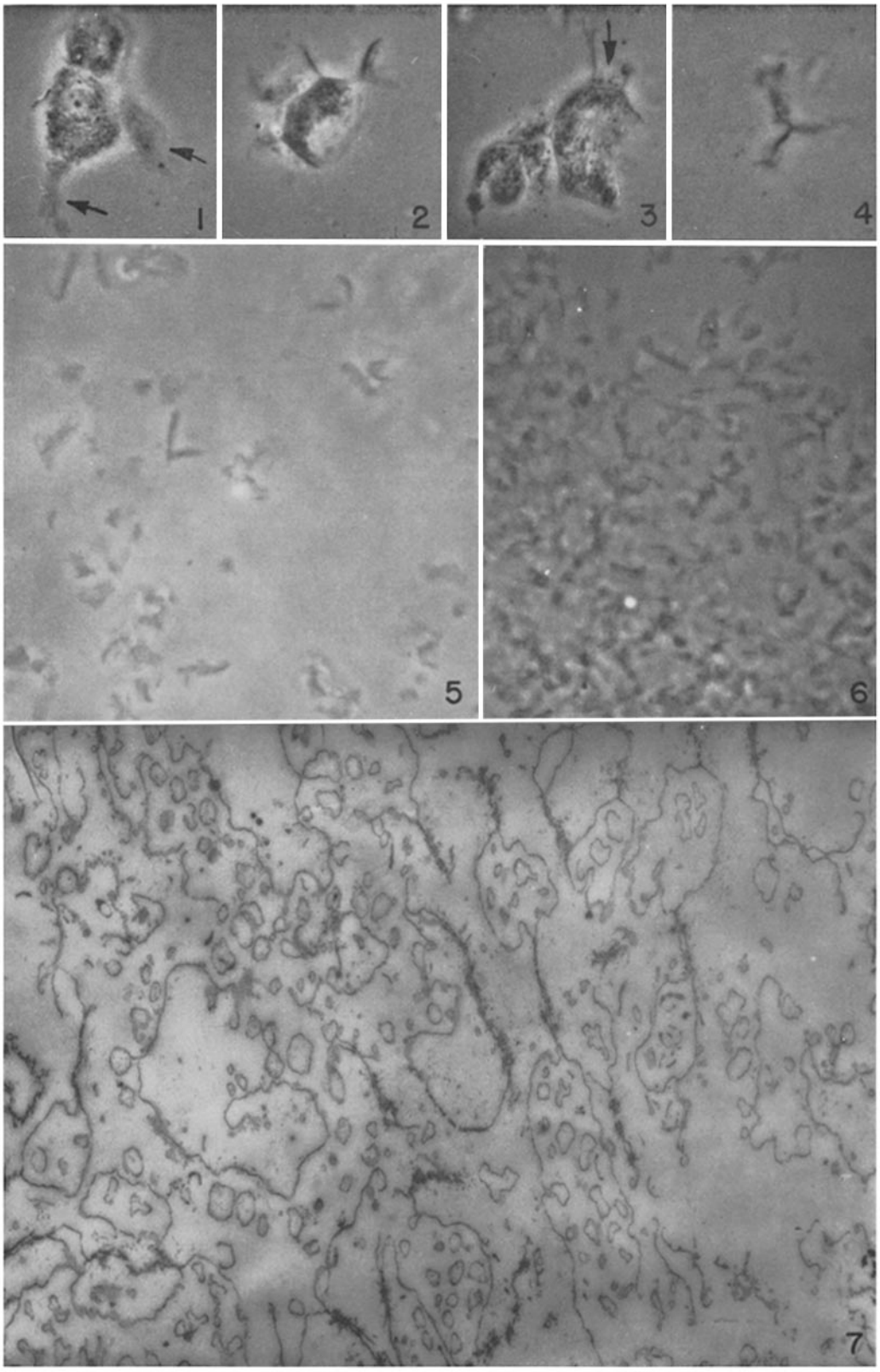
Liver cell membrane preparation. The membranes are seen as flat sheets or on edge. Only an occasional angulated configuration is present. This is due to the continual fragmentation of the membranes during the isolation procedures. By inspection many of these membranes can not be differentiated from nuclear membranes. The nuclear membranes have been eliminated by the homogenization in water and the flotation step. Whole nuclei and mitochondria are not seen.

FIGURE 6

Liver cell membrane preparation. Prior to embedding in methacrylate the cell membranes have been packed by centrifugation into a thin pellet the edge of which is seen near the upper border of the figure. The high concentration of the membranes allows for an estimation of the purity of the preparation which considering the homogeneity appears high.

FIGURE 7

Cell membrane preparation. Electron micrograph × 11,000. This is a representative section from the pellet. Much of the material is represented by closely parallel lines which separate into circular profiles and then again become opposed. Nuclei and mitochondria with their characteristic *cristae mitochondriales* are not seen. Microsomes with attached RNP particles are not identifiable. Many of the vesicles approaching microsomal size lie within the boundaries of the circular profiles similar in structure to bile canaliculi with villous interdigitations. Vesicles outside circular profiles probably represent villi of single membranes or torn bile canaliculi. Free-floating microsomes and mitochondria have been eliminated by centrifugation. Mitochondria and microsomes adherent to the membranes seen in earlier preparations isolated in a sucrose media are not seen in this preparation.



was the only one which prevented cytoplasmic particles from adhering to the sheets. Nuclear envelopes were noted to disappear after repeated homogenizations in water containing bicarbonate, but not in sucrose-containing media.

Observations on the Isolated Cell Membrane Fraction: In electron micrographs of sections of intact liver (Fig. 9), cell membranes of adjacent cells tend to be closely parallel and about 200 A apart. At intervals the membranes separate to form bile canaliculi with dimensions in the range of 5 to 10 μ. Irregular finger-like projections extend from the bounding cells into the lumen of the canaliculi. Where membranes of adjacent cells are closely apposed to one another, numerous small vesicles are frequently found within the cytoplasm of the hepatic cells close to the cell surface. Frequently, regions of diffuse high density material are present in these areas, presumably acting like the more highly organized "adhesion plates" or "desmosomes" found in other tissues (13).

Electron micrographs of the embedded and sectioned cell membrane fraction show details which bear a surprising resemblance to the intact tissue described above. Numerous, closely parallel, lines are present which separate at intervals to form structures of general dimensions and shape similar to the bile canaliculi of intact liver. Within these regions are a number of rather large vesicles which occasionally are continuous with one of the bounding membranes. At low magnifications (Fig. 7) there is seen to be granular, sometimes vesicular material lying close to the outward facing surfaces of the parallel membranes.

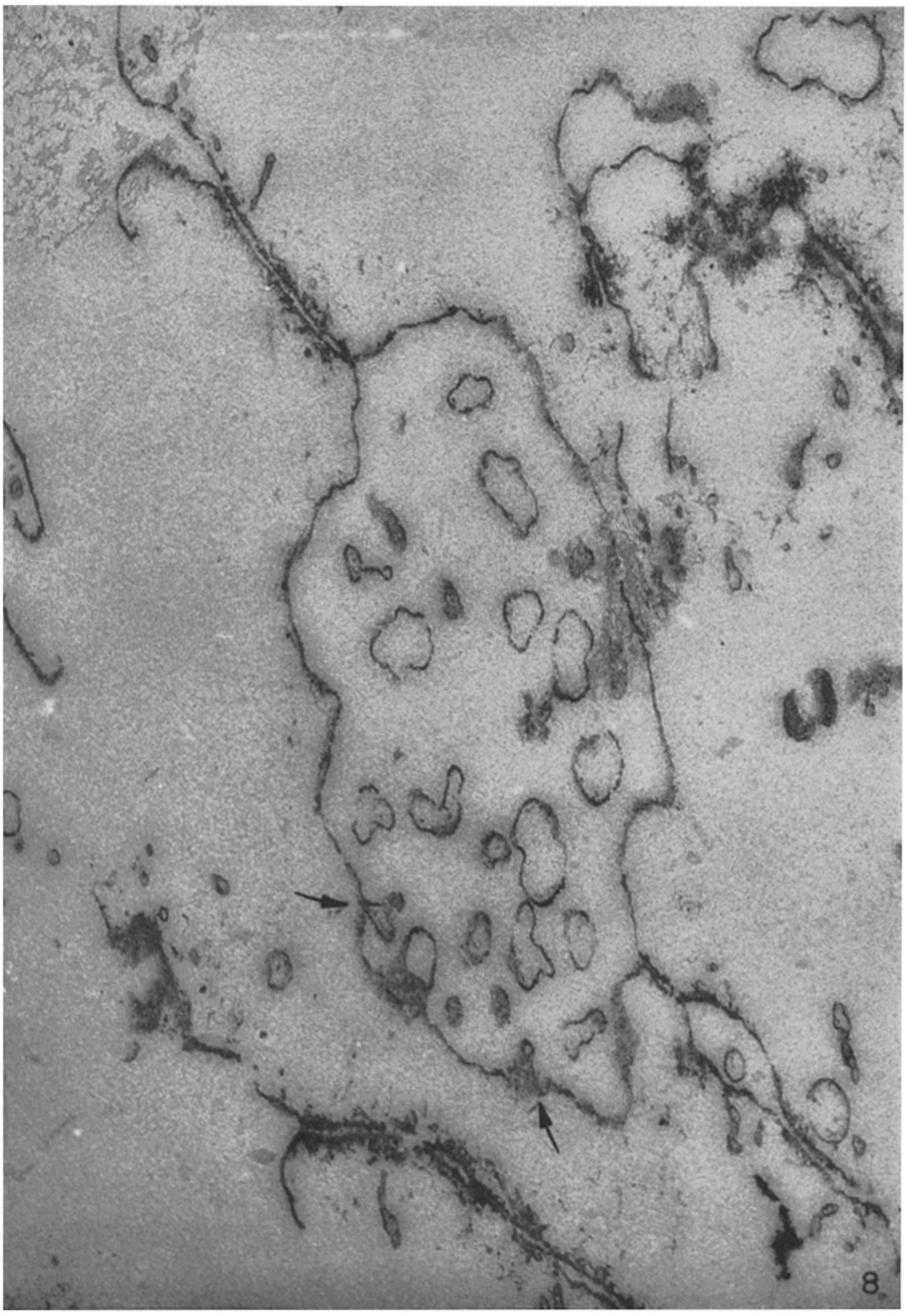
A characteristic feature of this preparation is the moderate number of single "open-ended" membranes, that is, membranes which are torn and do not close on themselves. In general, it appears that endoplasmic reticulum (14) and mitochondrial membranes (15) in water preparations do not tear in this fashion but tend to remain as closed vesicles. Mitochondria in water preparations are generally recognizable by the enclosed profiles of *cristae mitochondriales* which are not seen here. Some mitochondria may be present in unrecognizable form, but most of the membrane profiles are rather too large to be derived from them. Vesicles resembling microsomes with attached ribose nucleoprotein particles are not seen. The only vesicles of microsomal size generally lie within the circular boundaries of the membranes representing microvilli of the bile canaliculi. The finely granular dense material lying close to the outward facing surfaces of the closely opposed membranes resembling the desmosomes of other tissues is generally of too small a grain to represent RNP particles (Fig. 8). Examination of the light micrographs indicates that the cell membrane fraction is quite homogeneous and free from nuclei and mitochondria (Figs. 5 and 6). The membranes appear as thin amorphous sheets similar to those seen in the earlier stages of preparation except that they are smaller and generally lack their angulated structure. This is thought to be due to progressive fragmentation of the membranes during the isolation procedures.

DISCUSSION

One source of evidence indicating that the cell membrane fraction is of cell membrane origin is the observation of cell breakage, with separation of an identifiable cell membrane, which can be

FIGURE 8

Cell membrane preparation. Electron micrograph × 50,000. The profiles of adherent adjacent cell membranes are seen as represented by closely parallel lines ~200 A apart and ~150 A in thickness. The profile in the center of the plate splits forming a circular profile containing vesicular material formed by microvilli from the single membranes (see arrows). Compare this profile to the bile canaliculus in intact liver, Fig. 9. Note the absence of identifiable adherent microsomes or mitochondria. The finely granular dense material adherent to the membranes *only* in zones of close apposition may be similar to the "condensed cytoplasm" described in the desmosomes of other tissues.



followed through to the final preparation. Gradual fragmentation of the cell membranes results in some loss of the identifying "Y," "V," and "L" configurations, so that in the final preparation many single sheets are present which can not be distinguished morphologically from nuclear membranes. However, the great majority of nuclear membranes were noted to disappear during repeated homogenizations. The remaining are presumably sedimented during the flotation, previous work done in this laboratory suggesting that nuclear membranes isolated in a sucrose medium have a density greater than 1.22.

The other type of evidence indicating that the fraction is of cell membrane origin is the close similarity between the morphology of the isolated membranes and the structures generally regarded as cell membranes in intact tissue by electron microscopists. While these microscopic observations are qualitative, the fortunate presence of closely adhering membranes together with other details supports the idea that a substantial portion of this fraction consists of cell membranes. Since these membranes largely consist of two adherent membranes from adjacent cells and bile canaliculi, their major source is probably derived from the biliary or cell front as opposed to the blood front of the liver cell. The less frequently seen single "open-ended" membranes may represent contributions from the blood front or may simply be sections through torn bile canaliculi. The total absence of identifiable mitochondria, nuclei, and microsomes with attached RNP particles, indicates that major contamination with these particles is not present. The possibility that important constituents of the membranes are lost during isolation must be considered. Chemical, enzymatic, and refractometric measurements will probably be required to determine whether the membranes have a stable composition during isolation.

The term *cell membrane* as used in this paper represents the most peripheral, visible portion of the liver cell which has a membranous structure. This is a purely structural definition. What relationship this structure has to the functional "cell membrane," *i.e.*, the sum total of all those functions which segregate the intracellular environment from the outside and regulate the flow of materials into and out of the cell, remains to be elucidated. It is believed that the availability of a cell membrane fraction will provide an opportunity to investigate these structural and functional interrelationships of the cell membrane.

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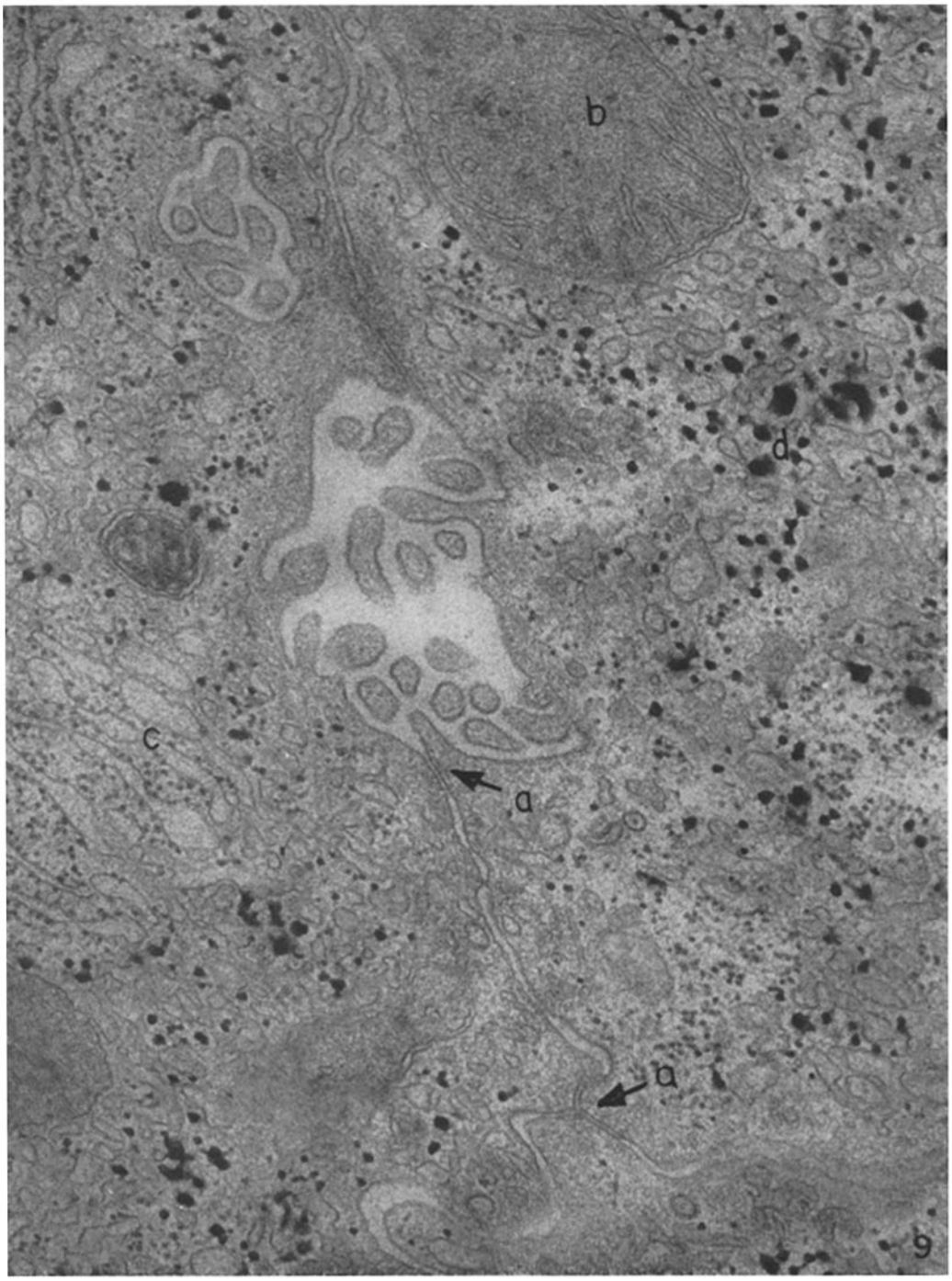
FIGURE 9

Intact rat liver. Electron micrograph × 50,000. The juncture of three adjacent liver cells is seen. The cell membranes of adjacent cells tend to be closely parallel and ~200 A apart. Zones of closer membrane apposition are seen where the membranes appear more dense (*a*). These areas may be similar to desmosomes where apparent increased membrane density results from a thin layer of finely granular dense material closely applied to the cytoplasmic membrane surface. This type of arrangement is seen in the isolated membranes, Figs. 7, 8. Near the center of the micrograph the membranes are seen to separate forming a bile canaliculus which contains microvilli from the free surface of the liver cell. Note the similarity between this structural arrangement and that in Fig. 8. Mitochondria (*b*), endoplasmic reticulum (*c*), and glycogen particles (*d*), are present and in close proximity to the membranes but are not identifiable in the cell membrane preparation.



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