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PLASMA MEMBRANES OF LIVER CELLS OF THE CHICK EMBRYO

I. ISOLATION PROCEDURES

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SUMMARY

The technique of isolation consists of a combination of homogenization, differential centrifugation, gradient centrifugation and the multiple poolings of isolated fractions. Large numbers of embryos are used to provide 5–10 g of accumulated liver tissue from the younger embryos (7, 11 days). Contamination by components of red blood cells or nuclear envelopes is avoided. The tissues are homogenized as dilute suspensions in either isotonic Krebs–Ringer solution or isotonic sucrose solutions supplemented with magnesium. Intact red blood cells are separated from the homogenate. Initial fractions are separated by multiple differential centrifugations. Fluffy coats and supernatants are pooled sequentially to increase the final yield of membranes. Intact nuclei can be separated by centrifugal rotation at 100 000 $\times$ *g* for 2 h over appropriate sucrose solutions. The separation of the plasma membrane fraction is carried out by density gradient centrifugation at 150 000 $\times$ *g* for 2 h. Fractions isolated in either isotonic sucrose or saline appear to be similar. The cytochrome oxidase and glucose-6-phosphatase activities are negligible. β-Glucuronidase activity is exceedingly low. Mg^{2+}-dependent ATPase activity is enhanced by the presence of Na^+. The isolation of plasma membrane of embryonic cells allows for a more direct assessment of membrane development, cell-contact phenomena and membrane function.

INTRODUCTION

Within the past few years several methods have been developed for isolation of the plasma membranes of cells. PALADE[1] and DOUNCE *et al.*[2] noted that several membranes, presumably plasma membranes, were concentrated in the fluffy layer above the nuclear fraction during differential centrifugation. NEVILLE[3] isolated from this fraction by sucrose-density gradient centrifugation what apparently is the plasma membrane. EMMELOT *et al.*[4–6] added additional discreet layers in the sucrose gradient to separate out the mitochondrial fraction. HERZENBERG AND HERZENBERG[7], TAKEUCHI AND TERAYAMA[8] and later COLEMAN *et al.*[9] made additional modifications through the use of isotonic sucrose as the suspending medium. These procedures were generally used for liver cells of the adult rat where large amounts of tissue could be obtained. WALLACH[10] has developed methods for the isolation of the plasma membrane of ascites tumor cells using Ficoll-density gradients. WARREN, GLICK AND NASS[11] first fixed the cell perifery with an agent such as $ZnCl_2$ and then removed the "hardened" cell perifery from the cytoplasm. Using osmotic shock of the mitochondrial fraction, DE ROBERTIS *et al.*[12] have isolated synaptic membranes. McCOLLESTER AND SEMENTE[13] noted the need to break down the cytoskeleton before the contents of skeletal muscle cell segments could be extracted. KUNO AND COLOWICK[14] isolated the membranes of mammalian muscle cells by repeated extractions with high ionic strength washes. O'NEILL[16] isolated the cell surface membranes of Amoeba proteins by extraction with high molarity sucrose followed by rupture and differential centrifugation. AKETA[16] has isolated the plasma membrane of the sea urchin egg by removing the fertilization membrane by immersion in a molar solution of urea followed by rupture of the eggs with outflow of ovoplasm.

In general, regardless of the technique used, the isolated fraction consists of membranes which exhibit the typical trilaminar structure under the electron microscope. Except for the brief notes of AKETA[16] and of ROSENBERG[17] few attempts have been made to isolate the plasma membranes of embryonic cells. The reasons are apparent. The yield of membrane fraction is very low. Embryos provide very small amounts of initial tissue. The fragility of the embryonic tissue differs considerably from that of the adult and the presence of any contaminant such as red blood cell membranes can cause very large errors in determining the purity of the final membrane yield. This paper describes methods which have been developed for the isolation of the plasma membrane of embryonic chick liver cells. The liver of the chick embryo was used as the test material, since fairly large numbers of fertilized eggs can be pooled and there is no need to be concerned with the effects of maternal diet on the lipid composition of the isolated membranes.

PROCEDURES AND MATERIALS

Tissue preparation

Fertilized chick embryo eggs were incubated for periods ranging from 7 to 21 days. The amount of tissue removed depended upon the age of the embryo. All tissue was removed within 2 h or less, placed in a chilled solution of isotonic balanced saline solution at 4°, and the homogenization procedure was started no more than 2 h after removal of the first pieces of tissue. For embryos where it was possible to remove larger units of tissue, homogenization could be started within a period of 0.5 h. The average wet weight of the liver tissue as a function of the age of the embryo is shown in Fig. 1. The increase in wet weight of the liver follows the expected sigmoid relationship. In general the smallest amount of tissue from which suitable membrane fraction could be obtained was approx. 5 g wet weight. Difficulties arise in obtaining sufficient amounts of tissue in the case of the 7-day-old embryo unless one pools fractions obtained at different periods of time. By the time the embryo is 11 days of age, it is quite easy to isolate within a 2-h period an appreciable amount of tissue, and as many as 450 embryos could be handled in this manner by 2 to 3 people.

Suspending media

In various experiments three different suspending media were tested. They will be referred to as Hanks balanced saline solution, Krebs Type 3 balanced saline solution, and isotonic sucrose. The compositions of the first two media are the same as those tabulated by PAUL[19] (1965) except that the Krebs Type 3 balanced saline solution is made up of 0.16 M sodium glutamate rather than 0.10 M glutamate as indicated in the reference. The isotonic sucrose consists of 0.25 M sucrose and 1 mM $MgCl_2$.

General procedure

All steps are carried out at 4°. The tissue is gently minced with a sharp scissors. Initial homogenization is carried out in a crude garlic press that can be purchased in

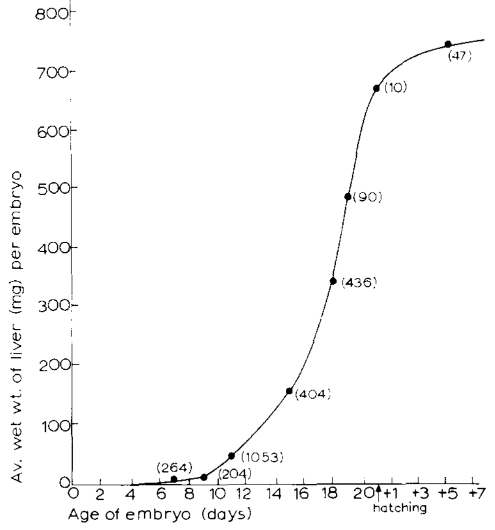

Fig. 1. Average weight of liver as function of age of embryonic and newborn chick. Number of embryos given in parentheses.

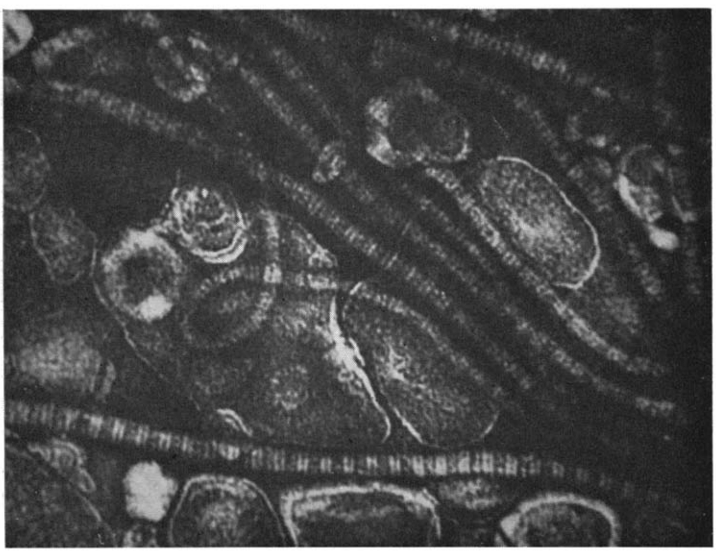

Fig. 2. Electron micrograph of phosphotungstic acid negatively stained membrane fraction contaminated with collagen matrix.

any houseware department. With continuous washings the cells are separated from capsular materials. If this step is omitted, the homogenate may contain several strands of collagen as shown by the electron micrograph of negatively stained material (Fig. 2). Further homogenizations require the use of a loose teflon pestle and glass mortar (200–300 μ clearance, 4–6 strokes). Sterile solutions are used to reduce bacterial contamination, and this precaution is especially important when sucrose solutions are used.

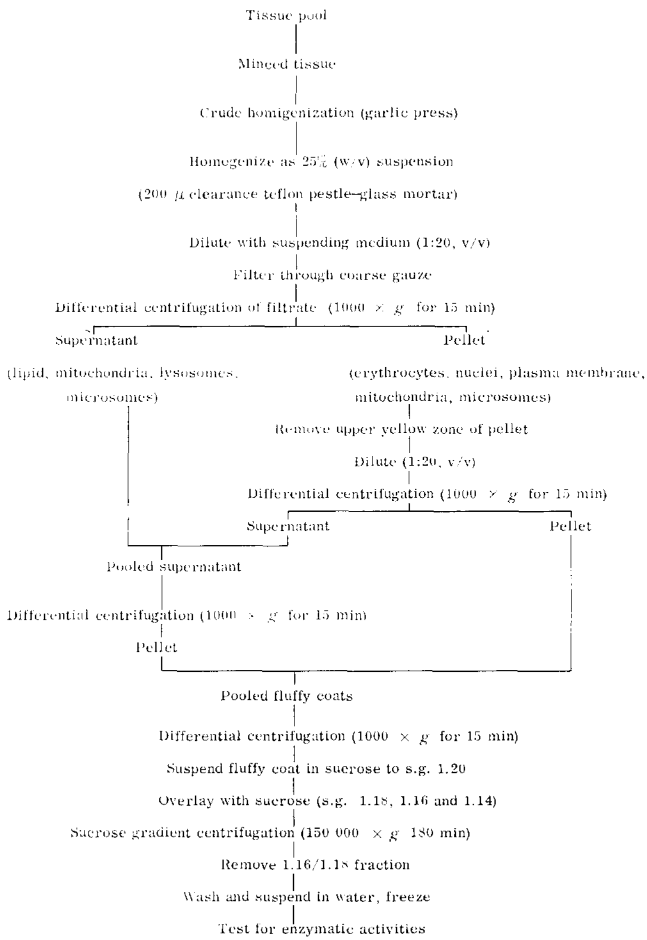

Fig. 3. Step-wise flow chart for isolation of plasma membranes.

The individual steps of the procedure are shown in the flow chart of Fig. 3. Hypotonic media and osmotic shock are avoided because of damage to red blood cells and to nuclei of cells. Red blood cell ghosts and nucleoprotein gels tend to mask the small amount of membrane being isolated (Fig. 4). As indicated on the flow chart, high-dilution factors are used for all steps to prevent trapping of materials among fractions.

Figs. 5 and 7 are representative fractions photographed with a microscope equipped with interference phase optics. During the first steps of differential cen-

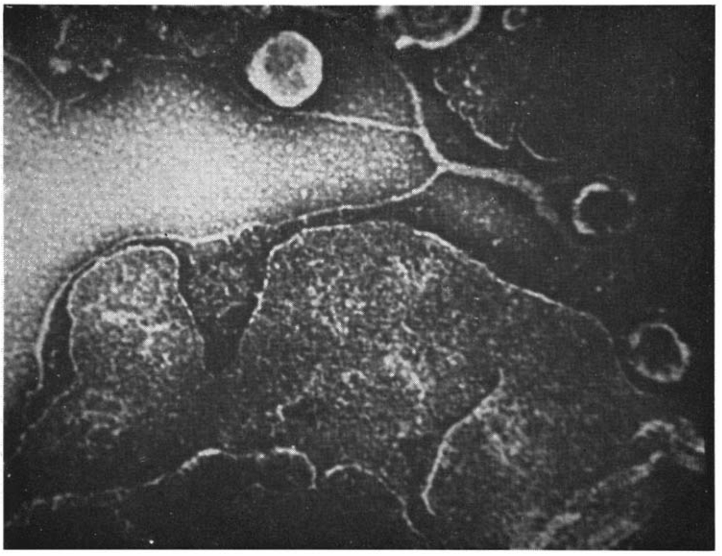

Fig. 4. Electron micrograph of phosphotungstic acid negatively stained membrane fraction contaminated with strands of DNA.

trifugation, three zones can be identified; a pellet, a yellowish fluffy coat and the supernatant fluid (Figs. 5a–5c). By the second or third step of differential centrifugation, including the pooling of zones, the fluffy coat takes on a more homogeneous appearance consisting mostly of intact nuclei, membrane fragments, mitochondria and some microsomal particles (Fig. 5d). The intact red blood cells are packed in the pellet.

Sucrose gradient centrifugation is carried out in a discrete four-step gradient. The successive measured specific gravities are 1.20, 1.18, 1.16 and 1.14. The fractions are spun in a Spinco Type SW 50L rotor at 150 000 $\times$ g for 180 min. Four fractions are obtained, namely, a pellet, a 1.18/1.20 fraction, a 1.16/1.18 fraction and a very

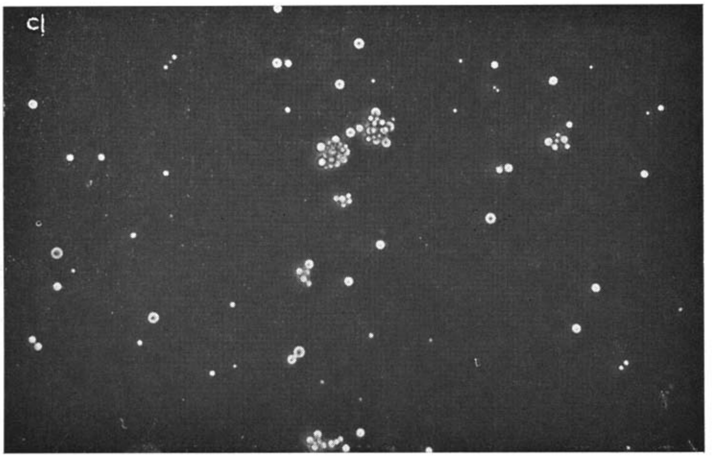

Fig. 5a. For legend see next page.

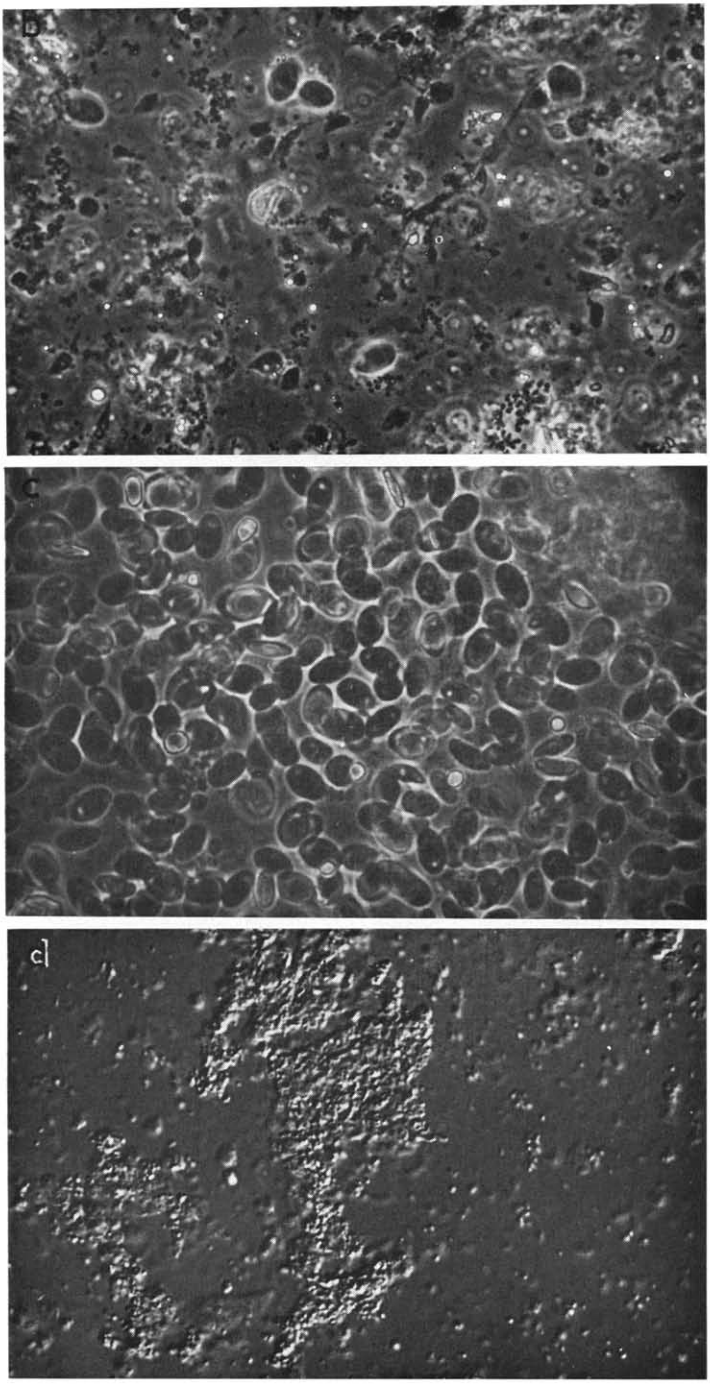

Fig. 5. Light micrographs of the fractions during stages of differential centrifugation. (a) lipid droplets of supernatant (1200 ×); (b) heterogeneous fluffy coat above pellet (1200 ×); (c) pellet during early differential centrifugation (1200 ×); (d) fluffy coat following several stages of differential centrifugation (1200 ×).

TABLE I

FURTHER PURIFICATION OF MEMBRANE FRACTION BY MULTI-STAGE SUCROSE GRADIENT ULTRACENTRIFUGATION

Plasma membrane fraction of 19-day-old embryonic chick liver	*Cytochrome oxidase (µmoles of reduced cytochrome oxidized per min per mg protein)*	
	First purification	*Second purification*
Saline isolation	22.8	0
Sucrose isolation	32.4	0

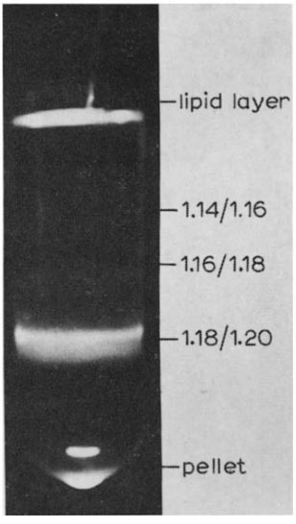

Fig. 6. Banding following sucrose-density gradient ultracentritugation.

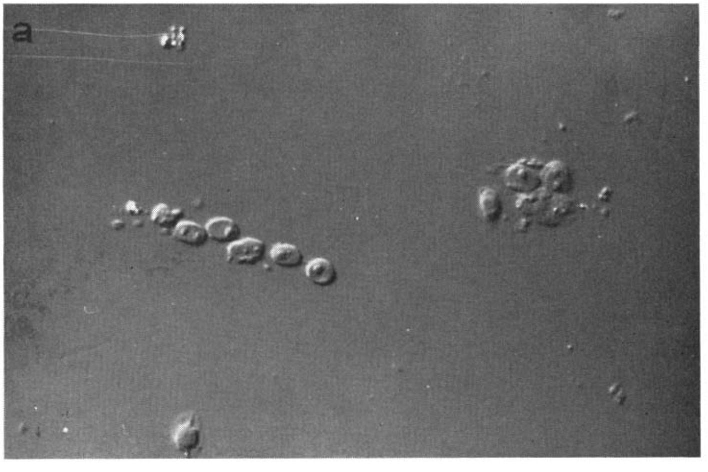

Fig. 7a. For legend see next page.

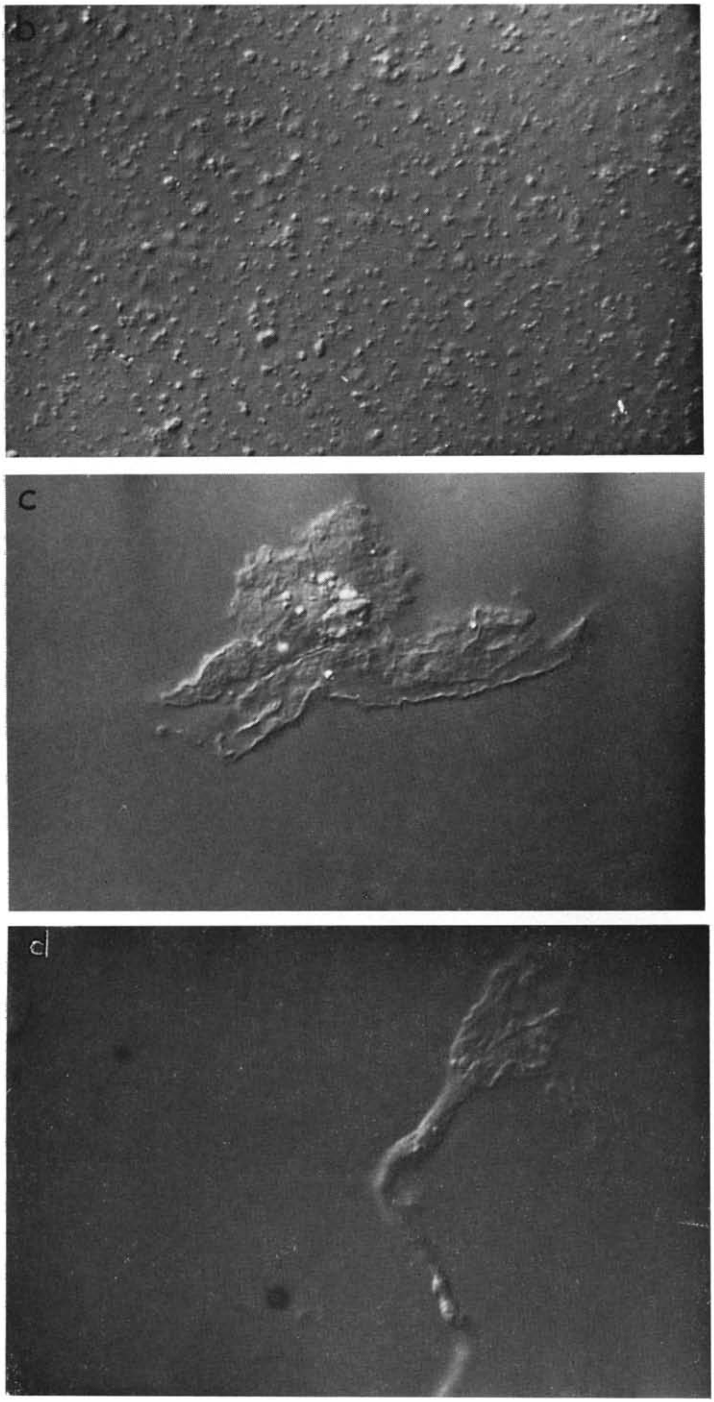

Fig. 7. Light micrographs of the fractions following sucrose-density gradient ultracentrifugation, (a) pellet of intact nuclei (1200 ×); (b) 1.18/1.20 fraction, mostly mitochondria (1200 ×); (c) and (d) 1.16/1.18 fraction, mostly plasma membrane (1600 ×).

small amount of 1.14/1.16. Insufficient dilution can result in trapping and must be followed by additional ultracentrifugation. An extreme example of trapping during the first gradient separation is shown by the measurement of cytochrome *c* oxidase in Table I.

Fig. 6 illustrates the banding of fractions following gradient centrifugation. Representative samples of the fractions are shown in Fig. 7. The pellet consists of intact nuclei. The 1.18/1.20 fraction is red in color and consists mostly of mitochondria.

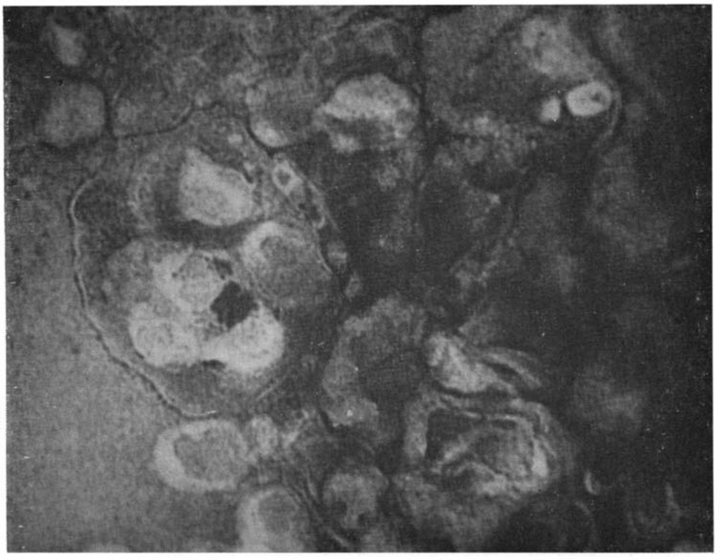

Fig. 8. Electron micrograph, phosphotungstic acid negatively stained preparation of plasma membrane fraction.

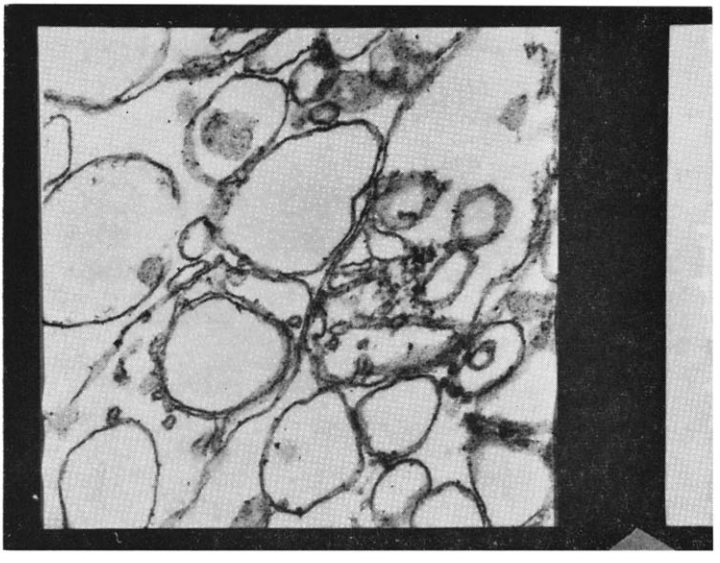

Fig. 9. Electron micrograph, O_sO_4 fixed and lead citrate stained section of plasma membrane fraction (47000 ×).

The 1.16/1.18 fraction is white in color and is made up of individual and aggregated membrane-bound particles and micelles intermixed with membrane fragments (Figs. 7c and 7d). Electron micrographs illustrate the membranous trilaminar aspect of this fraction (Figs. 8 and 9).

DISCUSSION

The use of isotonic saline or isotonic sucrose as the bathing medium during the fractionation procedure led to few differences in the fractions as observed by microscopy. No contamination by red blood cell ghosts or by adsorbed hemoglobin was noted. Both EMMELOT *et al.*[4] and COLEMAN *et al.*[9] refer to contamination of fractions by adsorbed hemoglobin. The higher viscosity of sucrose resulted in greater fragmentation of the material of the fluffy coat. Following sucrose-density gradient separation, both preparations yielded similar results. Some differences were noted in chemical analysis of the 1.16/1.18 membrane band as noted in Table II. Protein and total phosphate content are similar. Cytochrome *c* oxidase could not be detected. Glucose-6-phosphatase is very low. There was a slight amount of β-glucuronidase in the fraction homogenized in balanced saline, while none could be detected in that homogenized in isotonic sucrose. The (Na^{+}–Mg^{2+})-dependent ATPase activity of the fraction in Hanks balanced saline solution was approx. one-half that of the isotonic sucrose fraction but most of the ATPase activity in the fraction in Hanks balanced saline solution was ouabain sensitive, while only 6% of the APTase activity in the fraction in isotonic sucrose was ouabain sensitive. In both cases the fraction had been washed 3 times in double-distilled water (1:100, v/v) resulting in a loss of 60–80% of the protein.

Experiments in our laboratory have shown that cells suspended in various physiological media experience reduced loss of lipid in the presence of divalent cations and especially in the presence of Krebs Type 3 balanced saline solution. No differences were noted in the isolation procedure between Krebs Type 3 balanced saline solution and Hanks balanced saline solution. An approximate calculation indicated a double layer of the order of 100 Å for particles in isotonic sucrose and of the order of 10 Å for those in Hanks balanced saline solution. There appeared, however, to be only a slight

TABLE II

COMPARISON OF MEMBRANE FRACTIONS ISOLATED IN ISOTONIC BALANCED SALINE OR ISOTONIC SUCROSE SOLUTIONS

Plasma membrane fraction (*19-day-old embryonic chick liver*)	*Hanks balanced saline solution*	*Isotonic sucrose*
Protein (% of dry wt.)	10%	9%
Phosphate (μmoles/mg protein)	43.1	46.5
Cholesterol/phospholipid (molecular ratio)	1.0	—
Cytochrome oxidase (μmole reduced cytochrome *c*/min per mg protein)	0	0
β-Glucuronidase (μmole product/h per mg protein)	0.2	0
ATPase (Mg^{2+}) (μmole phosphate/h per mg protein)		
Na^{+}-dependent	31.9	64.1
Ouabain-sensitive (%)	83	6.3
Glucose-6-phosphatase (μmole phosphate/h per mg protein)	0.06	—

increase in particulate aggregation in Hanks balanced saline solution, and it did not interfere with the separation process. Apparently the Van der Waals attraction was sufficiently weak, such that it did not lead to difficulty in the isolation technique.

WIDNELL AND SIEKEVITZ[18], in studies of the plasma membranes of adult rat liver cells, treated the fluffy coat with versene prior to subjecting it to gradient ultracentrifugation. In similar fashion, tests were run on the effects of 1% versene on the separation process. It had no effect on the homogenization procedure with Hanks balanced saline solution. When the fluffy coat isolated in isotonic sucrose was washed with versene, no fraction could be recovered at the 1.16/1.18 band. The material was concentrated as a heterogeneous mixture at the 1.18/1.20 band. The reasons for this deviation in banding are not clear.

Several attempts were made to circumvent some of the tedious steps of the isolation procedure. The homogenate was passed through 1 μ filter mesh, but membrane fragments were not trapped. As a substitute for homogenization, cells were layered on a 30% sucrose solution where surface tension forces at the air–water interface fragmented the cells. Appreciable yields of membrane were not obtained. One additional modification proved useful on occasion. At an early stage of differential centrifugation, the fluffy coat was homogenized in a 54% sucrose solution. A 22% sucrose solution was layered above this. Centrifugation at 100000 $\times$ g for 120 min separated out intact nuclei in the pellet. The procedure outlined in Table I also yields a pellet fraction of intact nuclei.

Any isolation procedure can introduce several artifacts such as: (1) contamination by damaged red blood cells; (2) loss of soluble components; (3) structural rearrangement of the membrane; (4) desorption and resorption of components; (5) breakdown of fractions by hydrolytic enzymes; (6) trapping of subunits of the homogenate by nucleoprotein gels; (7) alteration in enzymatic activity due to variations in the H^+ concentration; (8) and autolysis of the fraction. The principle modifications which have been introduced in the procedures described above include the removal of intact red blood cells, the separation of intact nuclear and mitochondrial fractions, the pooling of membrane fractions and supernatants, and considerable dilution of the fractions to prevent trapping. The procedure appears to yield a fraction that consists mostly of plasma membrane and probably smooth endoplasmic reticulum. Isolation of these embryonic membranes should allow for a more direct assessment of membrane development, cell-contact phenomena and membrane function.

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