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THE ISOLATION AND CHARACTERIZATION OF THE PLASMA MEMBRANE FROM CHICK EMBRYO FIBROBLASTS

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SUMMARY

1. The plasma membrane has been isolated from chick embryo fibroblasts by a procedure involving differential and equilibrium density gradient centrifugations of untreated and zinc-treated cells.

2. The plasma membrane was isolated as large membrane sheets in the case of the zinc-treated cells and as smaller membrane vesicles and fragments from the untreated cells. Phosphohydrolases including the $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase (EC 3.6.1.3) and CTPase (EC 3.6.1.4) were concentrated in the plasma membrane fraction. This fraction was essentially free of RNA and the intracellular and mitochondrial membrane enzymes, NADH–cytochrome *c* reductase (EC 1.6.2.1) and succinate dehydrogenase (EC 1.3.99.1).

3. Sialic acids, cholesterol and phospholipid were concentrated in the plasma membrane preparations. The latter two components accounted for 65% by weight of the membrane.

INTRODUCTION

The plasma membrane serves the cell in a multiplicity of roles, from genetically controlled ion and metabolite transport[1,2] to providing specific surface recognition components which direct morphogenesis[3,4] and intercellular adhesions[3–5,52]. Changes in some of the functions of the cell membrane accompany the altered expression of the cell as it enters the oncogenic state. These changes have been expressed to varying degrees in altered growth requirements[6,7], modified cell metabolism[8], sometimes a loss of intercellular communication[9], decreased intercellular adhesions[10,11], and modified surface membrane chemistry[12–14].

An attempt to relate the oncogenic state to specific biochemical and morphological changes in isolated plasma membrane preparations has been the approach taken by some investigators who used cell systems such as rat liver and hepatoma[15–17], Ehrlich ascites carcinoma cells[18], HeLa cells[19,20], and L cells[21,22].

The Zn^{2+} method of WARREN *et al.*[23], with modifications, has been used for isolating very pure preparations of plasma membrane from cultured chick embryo fibroblasts. The results of this isolation method, along with the biochemical characterization of the isolated cell membrane, are presented in this communication.

MATERIALS AND METHODS

Fertilized chicken eggs were supplied by Sunnyside Hatchery of Oregon, Wisc. The modified minimal essential medium (Eagle's) was supplied by Schwarz BioResearch, and the calf serum from Grand Island Biological Co. Collagenase was obtained from Worthington. The phosphohydrolase substrates were purchased from Sigma or Calbiochem as the sodium or Tris salts.

Culturing and harvesting of cells

Primary cultures of chick fibroblasts were prepared from 13-day-old embryonated eggs by a method described by TEMIN[24] and were grown at 37° in a humidified CO_2 incubator in Eagle's minimal essential medium containing 6–10 % calf serum. The cells were transferred twice and cultured until they were a heavy, confluent layer. The cells were harvested by incubation for 10–15 min with 0.1 % collagenase (158 units/mg) in 0.16 M NaCl followed by scraping with a rubber policeman. The cells were suspended in 0.16 M NaCl, centrifuged, and washed twice by resuspension and centrifugation.

Isolation of the plasma membrane

Two methods were employed in the isolation and purification of the plasma membrane.

Zn^{2+} method. A modification of the Zn^{2+} method of WARREN *et al.*[23] was used for the isolation of large surface membrane fragments. To 1 vol. of resuspended chick fibroblasts at a concentration of $3 \cdot 10^8$ cells per ml were added 9 vol. of 0.001 M $ZnCl_2$ (pH 6.3). After 10 min at room temperature, 0.5 vol. of 1 % Tween-20 was added, followed, after 3 min, by 0.5 vol. of 0.01 M $ZnCl_2$. The suspension was cooled on ice and homogenized with a Potter–Elvehjem homogenizer until most of the surface membrane had been removed from the cells as determined by phase microscopy. The homogenate was spun at 200000 × *g* for 20 min and the resulting pellet suspended in 60 ml of 0.25 M sucrose. The suspension was centrifuged in the cold at 215 × *g* for 30 min in a clinical International centrifuge. The pellet was found to contain whole cells, cells which appeared to have had their surface membrane removed, and nuclei, with essentially no smaller debris. The supernatant contained fragments of membranes, cell debris, and a few nuclei and whole cells. The supernatants and resuspended pellets were treated with EDTA to a final concentration of 0.01 M (pH 7.5), incubated for 10 min at 4°, and centrifuged at 200000 × *g* for 20 min. The resultant pellets were suspended in 85 % (w/v) sucrose and brought to a refractive index of 1.430. Over this suspension was layered a linear 25–65 % (w/v) sucrose in water gradient (density = 1.094–1.241; total volume = 11 ml), and the material was centrifuged in a SW-41 rotor at 90000 × *g* for 16.5 h. The discrete bands of cellular components were removed, diluted with 0.16 M NaCl or water, and centrifuged. The pellets were washed twice by resuspension, dilution with saline or water, and recentrifugation. Certain of the fractions were purified on a second sucrose gradient by resuspending the washed pellets in sucrose to a refractive index of 1.392 and overlaying them with a linear 10–40 % (w/v) sucrose gradient (density = 1.038–1.150). The tubes were centrifuged at 90000 × *g* for 16.5 h, the layers removed, washed with saline or water, and saved for enzymatic or chemical analysis. In all experiments the

quantity of enzymes, phospholipid, RNA, *etc.*, put on the gradient was compared with their distribution in the numerous fractions. The term 'particulate homogenate' refers to the treatment of a homogenate with EDTA and the washes with saline and/or water; conditions which removed most of the soluble proteins.

Homogenization of untreated cells. The plasma membrane was also isolated from untreated chick embryo fibroblasts. Collagenase-dissociated cells were suspended in 0.16 M NaCl at a concentration of about $3 \cdot 10^8$ cells per ml and homogenized until all the cells were broken up. The conditions of EDTA treatment, gradient centrifugation, and washing were identical to those for the Zn^{2+}-treated cells.

Chemical procedures

(1) Protein was determined by the procedure of LOWRY *et al.*[25].

(2) The sialic acid content of the fractions was determined by the thiobarbituric acid procedure of WARREN[26], modified by the extraction of contaminants with isoamyl alcohol as employed by KRAEMER[27]. This procedure results in the loss of about 12 % of the sialic acid; the data presented in the tables have not been corrected for this loss.

(3) DNA was determined by the procedure of BURTON[28].

(4) RNA was determined by the method of FLECK AND MUNRO[29] as modified by BLOBEL AND POTTER[30].

(5) The carbohydrate content of the fraction was determined by the phenol–H_2SO_4 procedure of DUBOIS *et al.*[31], with glucose as a reference. This method does not detect hexosamine.

(6) Phospholipid and cholesterol were determined on a chloroform–methanol extract of the cell fractions[32]. Lipid phosphorus was determined according to the method of CHEN *et al.*[33]. A factor of 25 was used to convert μg phosphorus to μg phospholipid. Cholesterol was measured by the procedure of GLICK *et al.*[34].

Enzyme assays

The phosphohydrolase activity of intact cells and fractions was determined by incubating 25–100 μg of protein in a total volume of 1 ml containing 20 μmoles of *N,N*-bis-(2-hydroxyethyl)glycine (pH 7.8), 3 μmoles of substrate (in most cases CTP), 3 μmoles of $MgSO_4 \cdot H_2O$, and sucrose to a final concentration of 250 mosM. The reaction mixtures were incubated for 30 min at 37°. The quantity of P_i released from the substrates was measured by the procedure of MARTIN AND DOTY[35] as modified by LINDBERG AND ERNSTER[36], and the data are presented as μmoles P_i released per 30 min per mg protein. The enzymes studied were Mg^{2+}-ATPase (EC 3.6.1.4), $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase (EC 3.6.1.3), ADPase (EC 3.6.1.6), AMPase (EC 3.1.3.5), CTPase (EC 3.6.1.4) and CDPase (EC 3.6.1.6).

Mitochondrial contamination in the various cell fractions was determined by measuring the reduction of 2,6-dichloroindophenol at 37° in the succinate dehydrogenase–coenzyme Q reductase assay (EC 1.3.99.1) of ZIEGLER AND RIESKE[37].

The presence of intracellular membranes was evaluated by measuring the reduction of cytochrome *c* with NADH (EC 1.6.2.1) in the presence of 1 μg of antimycin at 37° (ref. 38).

Electron microscopy

The cellular fractions were prepared for examination with the electron microscope by fixing a suspension for 1 h with 1 % gluteraldehyde, 0.05 M cacodylate

and 0.2 M sucrose[39], followed by centrifugation, post-fixation in OsO_4, dehydration, and embedding in Epon–Araldite[40]. Thin sections were cut with glass knives, mounted on carbon-covered grids, and post-stained with lead citrate[41]. The sectioned material was examined in the Hitachi HU-11C electron microscope at 75 kV accelerating voltage and at instrument magnifications of 7500–40000.

RESULTS

The separation of cellular components

The isolation procedure using Zn^{2+} treatment of intact cells developed by WARREN *et al.*[23] gave a good preparation of surface membranes, although other methods have also been tried[42]. The procedure of WARREN has been modified by separating whole cells and nuclei from surface membrane fragments and debris with a clinical centrifuge and by the use of EDTA. The treatment of the pellet and the supernatant fractions with EDTA solubilized 68% of the original protein, but it was found to be essential for recovering phosphohydrolase activity and for dispersing the cellular membranes prior to their centrifugation on the sucrose density gradients. Initial experiments indicated that as much as 80% of the enzyme activity of an untreated sample could be recovered by removing the Zn^{2+} from the membranes with EDTA.

Equilibrium flotation centrifugation of the supernatant fraction produced the banding pattern A–D shown in Fig. 1a.

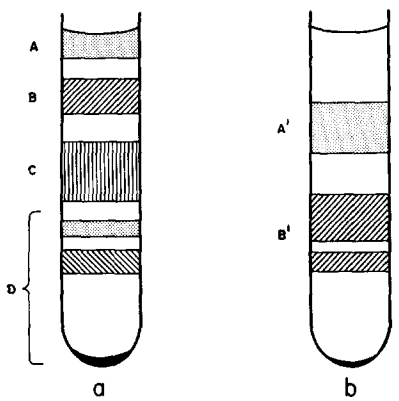

Fig. 1. The distribution of cell components on continuous density gradients of sucrose following equilibrium flotation centrifugation. a. Homogenate from untreated sample or supernatant fraction from Zn^{2+}-treated material on a 25–65% gradient. b. Redistribution of material in Bands A and B on a 10–40% gradient.

Biochemistry and morphology of Zn^{2+}-treated fractions

Low-speed centrifugation of the Zn^{2+}-treated homogenate sedimented virtually all free nuclei and intact and broken cells into the pellet fraction. DNA analysis of the supernatant and pellet fractions indicated that all measurable DNA was in the pellet fraction, with none detected in the supernatant. These data support the visual observations that only a few nuclei were present in the supernatant fraction. The membranes and organelles in the supernatant accounted for 34% of the particulate

TABLE I

THE DISTRIBUTION OF MEMBRANE-ASSOCIATED ENZYMES AND CHEMICAL COMPONENTS BETWEEN SUPERNATANT AND PELLET FRACTIONS FOLLOWING LOW-SPEED CENTRIFUGATION OF THE Zn^{2+}-TREATED HOMOGENATE

The percent distribution of the membrane components is given following separation of the homogenate (100%) into the supernatant and pellet fractions. The calculated means ± S.E. are based on the combined results of 5–13 experiments.

Component or enzyme	*Homogenate (units/mg protein)*	*Supernatant*		*Pellet*		*Recovery (%)*
		Units/mg protein	%	*Units/mg protein*	%	
CTPase (μmoles P_i released per 30 min)	2.44 ± 0.21	3.23 ± 0.28	42 ± 4	2.22 ± 0.19	30 ± 3	72
Cytochrome *c* reductase (μmoles reduced per min)	0.036 ± 0.008	0.026 ± 0.004	29 ± 9	0.057 ± 0.011	44 ± 9	73
Sialic acid (μmoles)	0.019 ± 0.002	0.025 ± 0.002	44 ± 5	0.017 ± 0.002	30 ± 5	74
Phospholipid (μg)	334 ± 22	496 ± 63	45 ± 10	407 ± 38	35 ± 6	80
Cholesterol (μg)	121 ± 13	127 ± 8.5	43 ± 8	91 ± 8	33 ± 7	76
RNA (μg)	99.4 ± 7.9	82.3 ± 9.6	40 ± 7	107.8 ± 10.1	39 ± 5	79
Carbohydrate (μg)	124 ± 13	256*		158*		
Protein (mg/ml)			34 ± 4		34 ± 3	68

* Based on 2 experiments.

TABLE II

THE DISTRIBUTION OF MEMBRANE-ASSOCIATED ENZYMES AND CHEMICAL COMPONENTS AMONG BANDS A CONTINUOUS DENSITY GRADIENT OF SUCROSE

The percent distribution of the membrane components is given following the separation of the supernatan experiments.

Component or enzyme	*Supernatant (units/mg protein)*	*Band A*	
		Units/mg protein	%
CTPase (μmoles P_i released per 30 min)	3.23 ± 0.28	8.52 ± 0.90	13 ± 2
Cytochrome *c* reductase (μmoles reduced per min)	0.026 ± 0.004	0.015 ± 0.005	2 ± 1
Sialic acid (μmoles)	0.025 ± 0.002	0.078 ± 0.009	17 ± 2
Phospholipid (μg)	496 ± 63	2158 ± 167	20 ± 2
Cholesterol (μg)	127 ± 63	761 ± 115	20 ± 2
RNA (μg)	82.3 ± 9.6	36.0 ± 5.9	2 ± 1
Carbohydrate (μg)	256*	419 ± 86	
Protein (mg/ml)		1.75 ± 0.37	5 ± 1

* Based on 2 experiments.

protein, and the specific activity or concentration of CTPase, sialic acid, phospholipid, and cholesterol was higher in the supernatant than in the pellet fraction (Table I). The first two parameters were considered specific for the plasma membrane. The specific activity of NADH–cytochrome *c* reductase was slightly reduced, together with that of RNA. Recovery of the various enzyme and membrane components was about 69–79 %.

Band A, obtained from the sucrose gradient, accounted for 5 % of the protein of the starting material, 13 % of the phosphohydrolase activity, and 17–20 % of the sialic acid, phospholipid and cholesterol (Table II). This fraction contained about 2 % of the supernatant's cytochrome *c* reductase and RNA. Band B, which accounted for 8 % of the protein, was also concentrated in CTPase activity, sialic acid, phospholipid and cholesterol. The specific concentrations and activities of RNA and cytochrome *c* reductase of Band B were less than those of the starting supernatant fraction. These latter components were concentrated in Bands C and D (Table II). The hexose and pentose content of the various fractions showed no distinctive distribution patterns in any of the experiments. Recoveries of the membrane components were between 65 and 90 %.

Electron microscopy of a thin section prepared from the A-band material revealed very long membrane fragments, some smaller vesicular membranes associated with the large sheets, but no mitochondria or membranes with ribosomes attached (Fig. 2). The material in Bands B, C and D was also membranous and vesicular (Fig. 3A, 3B and 3C, respectively). The B-band material contained vesicular membranes which did not appear to have free edges (Fig. 3A) and had a smaller diameter than plasma membrane vesicles prepared from untreated cells (Fig. 4). Mitochondria and granular-appearing membrane fragments and vesicles were present in the C band (Fig. 3B), whereas vesicular membranes, microfilaments and undefined cellular structures were found in the D band (Fig. 3C).

B, C AND D FOLLOWING CENTRIFUGATION OF THE Zn^{2+}-TREATED SUPERNATANT FRACTION ON A

(100 %) into the Bands A–D. The calculated means ± S.E. are based upon the combined results of 5–11

Band B		*Band C*		*Band D*		*Recovery* (%)
Units/mg protein	%	*Units/mg protein*	%	*Units/mg protein*	%	
5.76 ± 0.99	15 ± 2	3.30 ± 0.40	21 ± 2	1.46 ± 0.20	25 ± 3	74
0.029 ± 0.005	7 ± 1	0.062 ± 0.014	43 ± 11	0.0208 ± 0.003	43 ± 11	95
0.039 ± 0.002	14 ± 1	0.021 ± 0.003	16 ± 3	0.011 ± 0.001	18 ± 3	65
973 ± 105	15 ± 1	466 ± 41	19 ± 2	233 ± 16	28 ± 4	82
307 ± 51	12 ± 1	136 ± 28	14 ± 3	71 ± 8	21 ± 3	67
46.9 ± 7.8	3 ± 1	72.1 ± 4.9	10 ± 1	121.7 ± 12.5	54 ± 6	69
320 ± 85		439*		166 ± 12		
3.28 ± 0.73	8 ± 1	6.39 ± 1.46	18 ± 2	18.0 ± 3.1	46 ± 5	77

Biochemistry and morphology of untreated fractions

Experiments undertaken to study the banding patterns and biochemical distribution of membrane components from untreated homogenates established that these membranes and organelles banded in a manner identical to those from the Zn^{2+}-treated supernatant fraction (Fig. 1a). The overall distribution of protein, RNA and specific membrane components and enzymes among Bands A–D also remained about the same (Table III). The A band contained 6 % of the protein and about 20 % of the sialic acid, phosphohydrolase activity and phospholipid that was present in the starting material (Table III). The quantity of cholesterol in the A-band material had decreased from about 20 % in the Zn^{2+}-treated membrane to 15 % in the untreated membrane. The amount of RNA and NADH–cytochrome *c* reductase in the A band remained at the same level as observed with the Zn^{2+}-treated cells. Zn^{2+} treatment inhibited succinate dehydrogenase–indophenol reductase, the marker enzyme used to follow the distribution of mitochondria, and could not be employed in the earlier experiments. Assays for this enzyme with the untreated cells established that Band A was free of mitochondria, which were concentrated in the C band (Table III). The recoveries of the membrane components from the untreated cells were similar to those from the Zn^{2+}-treated cells, with the exception of cytochrome *c* reductase and succinate dehydrogenase, which were about 50 %.

The membranes in the A band obtained from the untreated homogenate were smaller in size than those from Zn^{2+}-treated cells, many were fragments with open edges, while others were vesicular (Figs. 4A and 4B). The B, C and D bands were in no way morphologically different from the bands obtained following Zn^{2+} treatment.

Biochemistry of refractionated A- and B-band material

The similar positions on the sucrose gradient and the biochemical properties of the material in Bands A and B suggest the presence of elements of a common

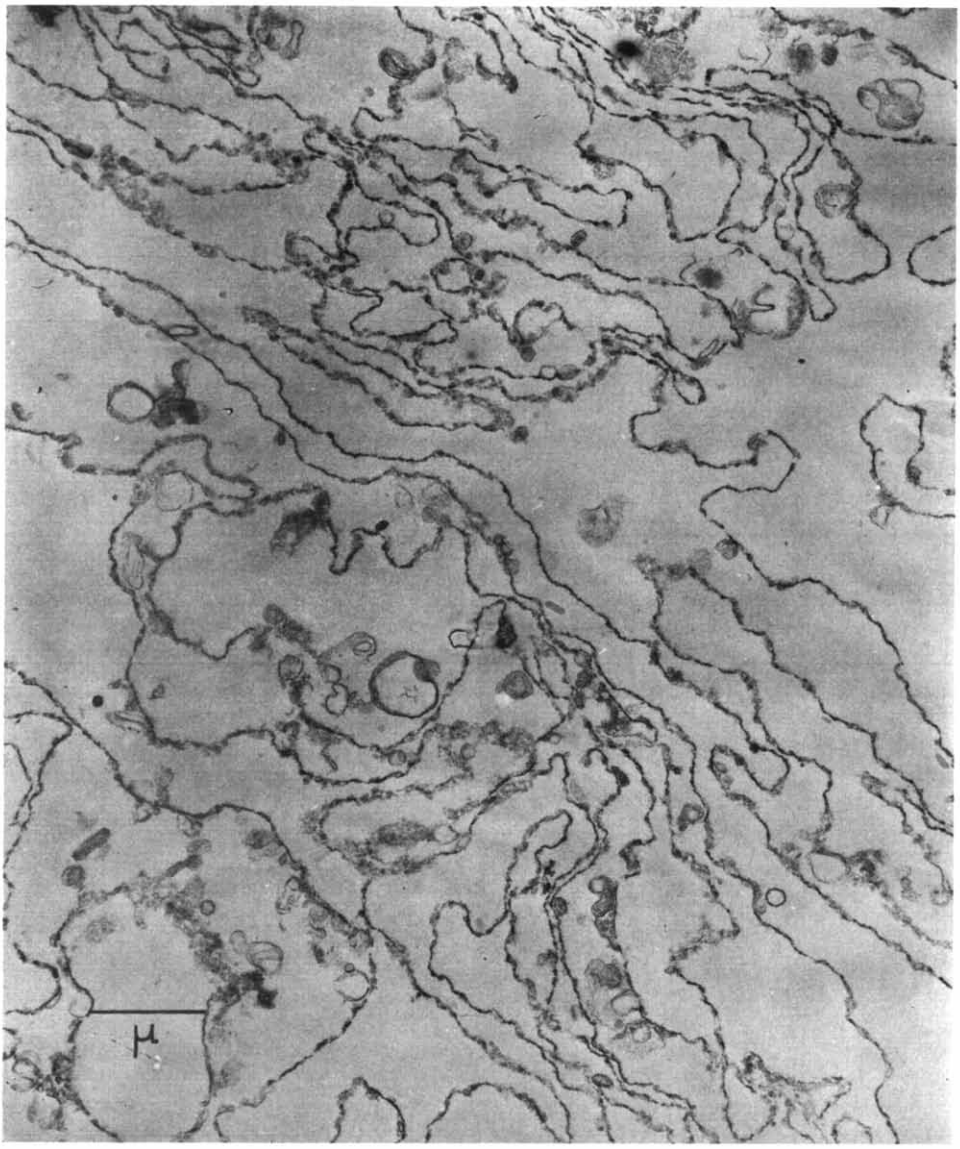

Fig. 2. A photoelectronmicrograph of a thin section prepared from a pellet of the A band. The plasma membranes from the Zn^{2+}-treated cells are isolated as large sheets that are free of cellular organelles and ribosome-associated endoplasmic reticulum. Membrane vesicles, either attached or entrapped, are also found with the isolated cell membrane. × 15000.

composition and membrane origin. When the membranes in these bands were separated and recentrifuged on sucrose gradients, separately or following mixing, the layering pattern illustrated in Fig. 1b from a mixed sample was observed. The original A-band material was distributed within the upper one-third of the centrifuge tube, designated as the A′ band, and was separated from a lighter lipid layer (Fig. 5) and from B-band material. The B-band membrane, centrifuged separately or after being

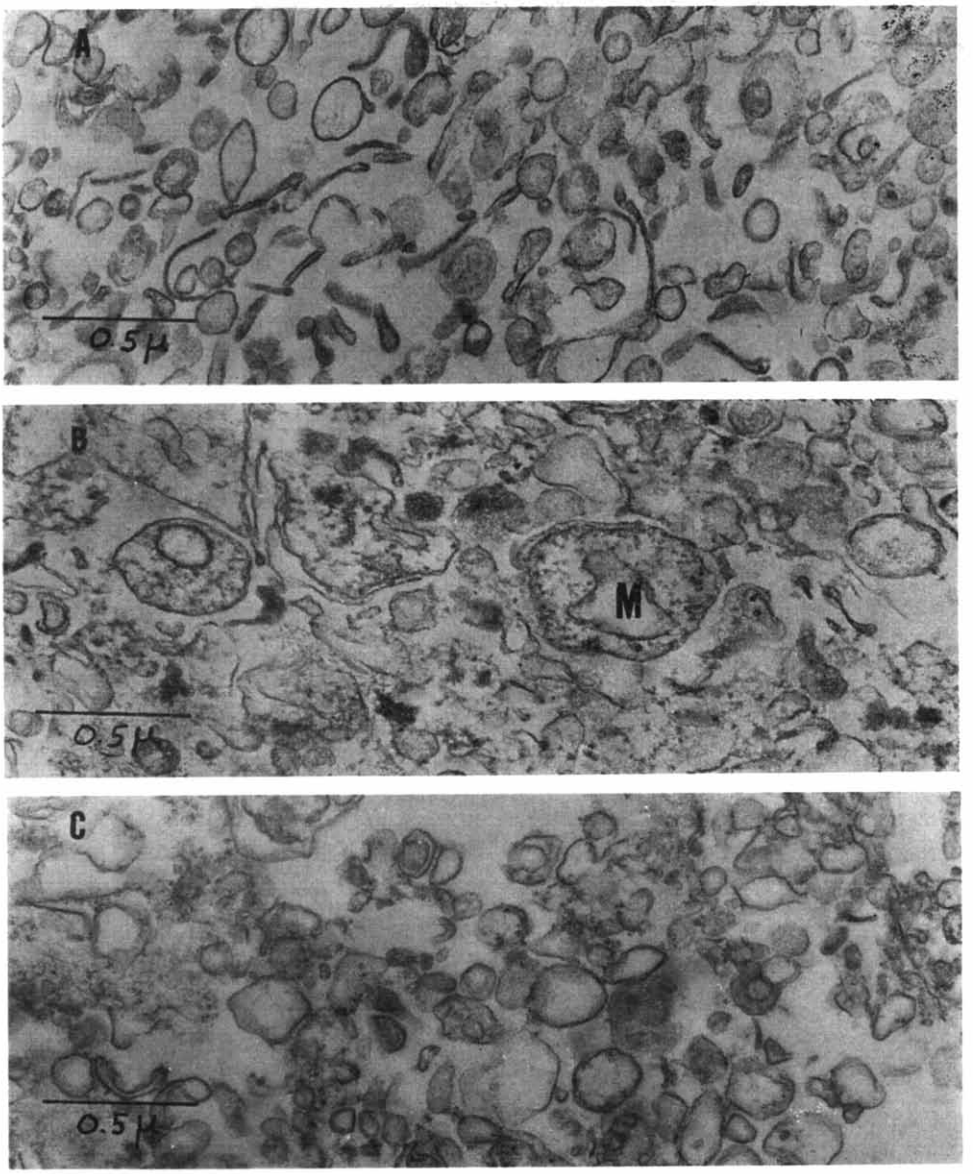

Fig. 3. Photoelectronmicrographs of thin sections prepared from pellets of the B, C and D bands. A. The membranes in the B band are vesicular and smaller in diameter than those observed in the A band from untreated cells (Fig. 4). There were no detectable mitochondria or "rough" endoplasmic reticulum associated with this fraction. × 40000. B. The membranes in the C band are more granular than those observed in the B band and contain swollen mitochondria (M). × 40000. C. The D band is a composite of discrete bands of cellular material and contains membrane vesicles, a few mitochondria, granular reticulum and microfilaments. × 40000.

mixed with the A band, was found in the lower two-thirds of the centrifuge tube and has been designated the B′ band (Fig. 5). B-band material always contained lighter membranes which rose into the A′-band region and components which were

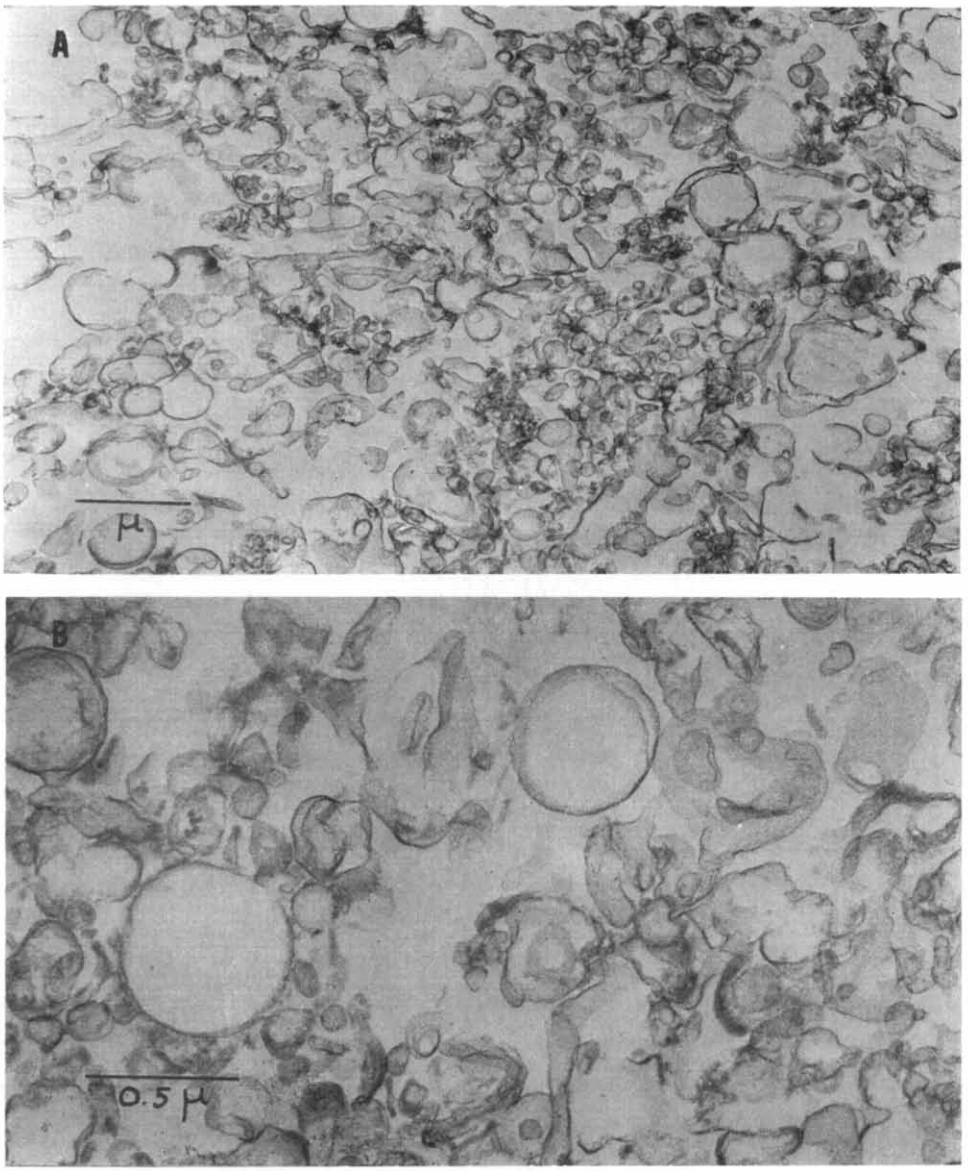

Fig. 4. Photoelectronmicrographs of thin sections prepared from a pellet of the A band. The plasma membranes from untreated cells are isolated in the form of fragments with open ends and as closed vesicles. A. × 15000. B. × 40000.

pelleted under these gradient conditions. The density of the A'-band material at equilibrium was 1.08, whereas that of the B' band was about 1.13.

The phosphohydrolase activity of refractionated A-band material, the A' band, did not increase (Table IV). The other plasma membrane components, notably sialic acid, phospholipid and cholesterol, increased in concentration as a result of the second centrifugation. The 2 % RNA and cytochrome *c* reductase that was present

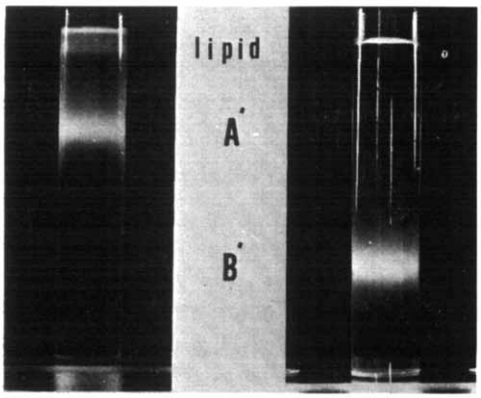

Fig. 5. The redistribution of membranes in the A and B bands following equilibrium flotation centrifugation in a continuous density gradient of 10–40% sucrose. The recentrifuged A-band material is found in the upper one-third of the tube, designated as the A′ band, while the B band is redistributed into the lower one-third of the centrifuge tube, the B′ band.

in the A band decreased to less than 0.5 %. The enzymatic and chemical components in the B-band membranes decreased in amount after centrifugation (Table IV). This was attributable in part to the dissociation and separation of less dense membranes into the A′ band.

The phosphohydrolase activity of purified plasma membrane

Unpublished studies[43] indicated that certain nucleotide tri- and diphosphatases, notably ADPase, CTPase, CDPase and UTPase, were associated with the cell surface of the chick fibroblasts. A comparison was made of the specific activities of these enzymes in a particulate homogenate and in the A-band fraction obtained from untreated cells. These enzymes, along with AMPase and the ouabain-sensitive, $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase, were concentrated from 3–5-fold in the isolated plasma membrane preparations (Table V).

DISCUSSION

A membrane preparation with morphological features identical to that of plasma membrane obtained from Zn^{2+}-treated L cells[23] is present in the A band. The membranes in this band, which accounted for 5 % of the protein of the starting material, (Tables II and III) are enriched in sialic acid. The amount of sialic acid in the membranes of the A and B bands accounted for as much as 50 % of the carbohydrate put on the gradient (Table III). When the membranes in the A band were purified further on a second gradient (Fig. 1b), the sialic acid concentration of the A′-band membranes increased to 0.1 μmole/mg membrane protein (Table IV). This value is the highest reported concentration of sialic acid within a membrane fraction. EMMELOT *et al.*[15] and BENEDETTI AND EMMELOT[17,44,53] have reported that the plasma membrane from rat liver and the hepatoma 484 containing 0.033 and 0.047 μmole of sialic acid/mg protein, respectively.

About 20 % of the phospholipid and cholesterol in the starting material was associated with the membranes in the A band (Tables II and III). This is a 5-fold

TABLE III

THE DISTRIBUTION OF MEMBRANE-ASSOCIATED ENZYMES AND CHEMICAL COMPONENTS AMONG BANDS GRADIENT OF SUCROSE

The percent distribution of the membrane components is given following the separation of the non- ± S.E. are based upon the combined results of from 4–8 experiments.

Component or enzyme	*Homogenate (units/mg protein)*	*Band A*	
		Units/mg protein	%
CTPase (μmoles P_i released per 30 min)	2.85 ± 0.36	10.84 ± 2.84	23 ± 7
Cytochrome *c* reductase (μmoles reduced per min)	0.046 ± 0.008	0.026 ± 0.006	2 ± 1
Succinate dehydrogenase (μmoles indophenol reduced per min)	0.006 ± 0.003	0.000	0.0
Sialic acid (μmoles)	0.023 ± 0.002	0.055 ± 0.003	19 ± 6
Phospholipid (μg)	368 ± 39	2186 ± 285	22 ± 5
Cholesterol (μg)	95 ± 12	573 ± 133	15 ± 2
RNA (μg)	89 ± 10	18 ± 14	1 ± 1
Carbohydrate (μg)	133 ± 9	266 ± 66	13 ± 4
Protein (mg/ml)		1.8 ± 0.6	6 ± 2

concentration of these components over that of the original particulate homogenate. The phospholipid content of the refractionated A′ band was 1900 μg/mg protein. Some of the lipid present in the A band from the first gradient was separated from the membrane on the second gradient (Fig. 5). This lipid may have been free lipid adsorbed to the membranes or may represent degradation and release of the surface membrane lipid during the preparative procedures.

An increasing ratio of cholesterol to phospholipid has been cited as a criterion of an isolated membrane[19,45]. However, calculation of the cholesterol:phospholipid ratio for the membrane fraction indicated that no differential enrichment of the A-band membranes occurred. A ratio of 0.5 and 0.7 was calculated for the two A-band membrane preparations. Values of 1.1 have been reported for myelin[45], 1.15 for erythrocytes[45,46], 1.05 for HeLa cells[19], 0.69 for L cells[22] and 0.38–0.7 for preparations of isolated liver cell membranes[44,47].

The distribution of the A- and B-band membranes on the second gradient also reflects the high lipid content of these fractions. The A-band membrane rises to a sucrose density of 1.08, whereas the B′ band is found in the 1.12–1.14 sucrose density region. These densities are much less than reported for the isolated plasma membranes of liver cells. These membranes sedimented to a sucrose density of 1.16–1.18 (refs. 15, 44, 47–49, 53).

The carbohydrate content of the membrane in the A band was 7–9 % by weight and was a reflection of the content of sugars other than hexosamine. There was no characteristic distribution of carbohydrate among the bands A–D (Tables II and III).

The phosphohydrolases, CTPase, ADPase, CDPase and AMPase, and the $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase were concentrated in the membranes of the A band (Tables II–V). The specific activity of CTPase was at its highest in the A band and decreased progressively in the lower bands. The failure to achieve an increase in

A, B, C AND D FOLLOWING CENTRIFUGATION OF AN UNTREATED HOMOGENATE ON A CONTINUOUS DENSITY

Zn^{2+}-treated particulate homogenate (100%) among the Bands A, B, C and D. The calculated means

Band B		*Band C*		*Band D*		*Recovery* (%)
Units/mg protein	%	*Units/mg protein*	%	*Units/mg protein*	%	
9.53 ± 2.08	38 ± 4	3.98 ± 0.57	18 ± 5	1.07 ± 0.25	7 ± 2	86
0.045 ± 0.013	9 ± 3	0.088 ± 0.018	28 ± 8	0.018 ± 0.001	15 ± 5	53
0.006 ± 0.001	4 ± 1	0.030 ± 0.006	28 ± 8	0.005 ± 0.001	16 ± 5	48
0.047 ± 0.003	31 ± 2	0.022 ± 0.002	14 ± 4	0.007 ± 0.001	12 ± 4	76
1057 ± 99	29 ± 2	499 ± 55	13 ± 3	194 ± 17	25 ± 6	89
345 ± 37	27 ± 4	117 ± 8	11 ± 3	53 ± 7	14 ± 2	67
48 ± 6	10 ± 4	54 ± 7	12 ± 4	104 ± 15	47 ± 19	70
177 ± 26	9 ± 3	177 ± 22	9 ± 1	353 ± 51	44 ± 13	75
5.1 ± 1.6	16 ± 3	5.5 ± 1.3	15 ± 4	20.7 ± 4.1	32 ± 5	69

TABLE IV

THE DISTRIBUTION OF MEMBRANE-ASSOCIATED ENZYMES AND CHEMICAL COMPONENTS BETWEEN BANDS A′ AND B′ FOLLOWING RECENTRIFUGATION OF THE A AND B BANDS ON A CONTINUOUS DENSITY GRADIENT OF SUCROSE

The calculated means ± S.E. are based upon the results of 5–12 experiments with combined data from untreated and Zn^{2+}-treated cells.

Component or enzyme	*Band A (units/mg protein)*	*Band B (units/mg protein)*	*Band A′ (units/mg protein)*	*Band B′ (units/mg protein)*
CTPase (μmoles P_i released per 30 min)	7.58 ± 1.82	9.43 ± 1.96	7.54 ± 1.63	6.99 ± 1.45
Cytochrome *c* reductase (μmoles reduced per min)	0.022 ± 0.004	0.044 ± 0.012	0.009 ± 0.002	0.022 ± 0.004
Succinate dehydrogenase (μmoles indophenol reduced per min)	0.000	0.007 ± 0.002	0.000	0.000
Sialic acid (μmoles)	0.061 ± 0.005	0.047 ± 0.004	0.100 ± 0.007	0.053 ± 0.005
Phospholipid (μg)	1440 ± 296	1116 ± 78	1875 ± 231	876 ± 80
Cholesterol (μg)	544 ± 92	388 ± 39	770 ± 34	374 ± 59
RNA (μg)	32 ± 13	40 ± 7	12 ± 6	39 ± 8
Carbohydrate (μg)	230 ± 73	254 ± 24	277 ± 59	274 ± 60

the concentration of the CTPase when the A band was recentrifuged on the second sucrose density gradient (Table IV) may possibly be owing to enzyme denaturation, since the other surface membrane components were increased in this fraction. WALLACH AND ULLREY[50] had observed that a low density membrane fraction derived

TABLE V

THE PHOSPHOHYDROLASE ACTIVITIES OF THE PARTICULATE HOMOGENATE AND THE MEMBRANE IN BAND A

Membrane fractions were incubated in a 1-ml volume containing 3 μmoles $MgSO_4$, 3 μmoles of nucleotide substrate, 18 μmoles Bicine (pH 7.8) and sucrose to 250 mosM. The $(Na^+ + K^+)$-stimulated Mg^{2+}-ATPase was assayed in the presence of 80 μmoles Na^+, 10 μmoles K^+ and 0.5 μmole of ouabain. The calculated means $\pm$ S.E. are based on the results of 3 experiments.

Substrate	*Homogenate (H)*	*Band A*	*Ratio A/H*
ATP	4.9 ± 1.1	11.1 ± 1.4	2.3
ATP + Na^+	4.5 ± 1.0	8.6 ± 0.8	
ATP + Na^+ + K^+	5.1 ± 0.6	11.2 ± 2.1	
ATP + Na^+ + K^+ + ouabain	4.4 ± 0.9	7.9 ± 1.2	
Difference − or + ouabain	0.7	3.3	4.7
ADP	1.4 ± 0.1	4.0 ± 0.3	2.9
AMP	0.6 ± 0.1	3.3 ± 0.5	5.5
CTP	1.9 ± 0.2	7.8 ± 1.4	4.1
CDP	0.9 ± 0.03	2.8 ± 0.5	3.2

from Ehrlich ascites cells was concentrated in ADPase and AMPase activites concomitant with an increase in the concentration of phospholipid and sialic acid, and WATTIAUX-DE CONINCK AND WATTIAUX[48] have recently reported that an ADPase was associated exclusively with the isolated plasma membrane from rat-liver cells.

An approximate composition has been calculated for the membrane in the A band based on the data in Tables II, III and IV. These membranes are made up of 25 % protein, 47–54 % phospholipid, 17 % cholesterol and about 7–8 % carbohydrate (Table VI). The plasma membrane isolated from other sources (liver[18,44,45,47,53], L cells [22], HeLa cells[19] and red blood cells[46]) had about 40 % lipid and 60 % protein. The RNA was less than 0.5 % in the purest preparation, the A′ band.

The membranes in the A band are not mitochondrial in origin, since succinate dehydrogenase activity could not be measured in this preparation. The enzyme

TABLE VI

THE CALCULATED COMPOSITION OF A PARTICULATE HOMOGENATE AND THE A- AND A′-BAND MATERIAL FROM UNTREATED AND Zn^{2+}-TREATED CELLS

The concentrations of all the components, expressed as μg/mg protein as presented in Tables I–IV, were added to 1 mg protein to obtain a composition which was equated to 100 %. A molecular weight value of 308 was used to convert μmoles sialic acid to μg sialic acid.

Component	*Homogenate* (%)	*A band* (%)		*A′ band* (%)
		Untreated	*Zn^{2+}-treated*	
Protein	59.4	24.6	22.7	25.2
Sialic acid	0.4	0.4	0.6	0.8
RNA	5.9	0.5	0.8	0.3
Phospholipid	19.8	53.8	49.1	47.3
Cholesterol	7.2	14.1	17.3	19.4
Carbohydrate	7.3	6.6	9.5	7.0

NADH–cytochrome *c* reductase was also at a low level of activity in both the A- and B-band membranes. This enzyme is generally considered a marker for the endoplasmic reticulum and possibly the outer membrane of the mitochondria. However, VASSILETZ *et al.*[51] have reported that isolated preparations of rat-liver plasma membrane contain electron transport components, one of which is NADH–cytochrome *c* reductase. The presence of low levels of reductase in the A band does not support their observations. It is likely that the enzymatic activity in our preparation is owing to contamination with intracellular membranes. However, the poor recovery of reductase activity in the untreated preparations (Table III) and the failure to increase the CTPase activity in the A′ band (Table IV) could argue for the position that the reductase is associated with the membranes in the A band but was denatured during its isolation.

The membranes of low density that are present in the A band represent a very pure preparation of plasma membrane. This plasma membrane is unique because of its high lipid content, but its other properties are similar to those reported for other cell membrane preparations. The use of collagenase to release the fibroblasts from the culture dishes does not result in a loss of sialic acid from the cell surface. However, it is possible that this enzymatic treatment, the EDTA addition and the saline or water washes remove surface membrane components of which we are unaware. With these limitations, a plasma membrane preparation of a high degree of purity can be prepared from cultured cells. These isolation techniques should make it possible to quantitatively measure differences in the composition of cell membranes from chick fibroblasts and from similar cells converted by the oncogenic RNA viruses[6,7]. Studies using this experimental design are in progress.

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