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THE ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANES FROM CULTURED CELLS

II. THE CHEMICAL COMPOSITION OF MEMBRANE ISOLATED FROM UNINFECTED AND ONCOGENIC RNA VIRUS-CONVERTED PARENCHYMA-LIKE CELLS

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SUMMARY

1. Cell membrane was isolated by flotation equilibrium centrifugation of a homogenate from cultured epithelial cells derived from rat liver, BRL cells, and from BRL cells which had been converted by Murine Sarcoma Virus (MSV).

2. On continuous density gradients of sucrose, the plasma membrane was localized in two fractions, the A′ band and the B′ band. Phospholipid, cholesterol, sialic acid, neutral and amino sugar, CTPase (EC 3.6.1.4), and AMPase (EC 3.1.3.5) were concentrated in the isolated membranes from the BRL cells.

3. Plasma membrane from the MSV-converted BRL cells was similar to that of the BRL cells in chemical composition but was markedly deficient in nucleotide di- and triphosphatases. AMPase, which was very high in the BRL cells, was increased still further in the virus-converted cells.

INTRODUCTION

The method of flotation equilibrium centrifugation of homogenates of chicken embryo fibroblasts[1] and chicken embryo fibroblasts converted by RNA sarcoma and leukemia viruses[2] has resulted in the isolation of plasma membrane of a high degree of purity. Biochemical and enzymatic evaluation of these preparations has established differences between membranes isolated from normal cells and those from the converted cells. These differences were mainly in the quantity of carbohydrate that is present in the membranes and may be related to shape change and decreased adhesiveness of the converted cells produced by changes in quantity and/or composition of intercellular cementing substances[3]. Unfortunately, chick fibroblasts can not be cultured as monolayers, and the number of junctional complexes formed between adjacent cells are not numerous. Therefore, the amount of intercellular cementing

Abbreviation: MSV, Murine Sarcoma Virus.

substance associated with the cell membrane from the fibroblasts may also be low.

Epithelial cells can be cultured as monolayers, they have specialized junctional complexes between cells, and they maintain strong adhesions with the substratum. The presence of a large number of intercellular contacts and the possibility that these junctions may have a distinctive chemical composition prompted attempts to isolate the cell membrane from epithelial cells by flotation equilibrium centrifugation[1,2]. The epithelial cells used in this study were from a line of rat liver parenchyma-like cells obtained from Dr. Hayden Coon. A line of Murine Sarcoma Virus (Harvey) converted cells has been developed from these parenchyma-like cells by TEMIN[4]. The isolation of plasma membrane from these cells and a comparison of the chemical properties of this membrane with those of the parenchyma-like cell membrane was initiated. The results of these studies established the feasibility of using flotation equilibrium centrifugation as a means of isolating plasma membranes from cultured epithelial cells. The plasma membrane from the parenchyma-like cells is similar in composition to that obtained from chick fibroblasts. The most distinctive difference between normal and virus-converted cell membrane is that the latter has a decreased ability to hydrolyze nucleotide di- and triphosphates but has higher AMPase activity.

MATERIALS AND METHODS

Cultures of rat liver parenchyma-like cells were obtained from V. R. Potter and are a cell line, BRL-3A, which was developed by H. G. Coon (see ref. 5) of the Carnegie Institute of Washington from cells obtained from the liver of a 5-week-old female Buffalo rat in February and given to Potter in March, 1968. These cells were mass-cultured, frozen, and stored in liquid N_2. Thawed and recultured BRL cells were used in experiments carried out over the past 12 months. These cells were converted by Murine Sarcoma Virus (MSV) (Harvey) by H. M. Temin (see ref. 4), who kindly provided us with these virus-converted cells. The modified minimal essential medium (Eagles) was supplied by Schwarz-Mann, and the calf serum by Microbiological Associates.

Culture and harvesting of cells

The BRL cells were grown at 37° in a humidified CO_2 incubator in modified Eagles minimal essential medium (Temin) containing 6–10 % calf serum. The cells were grown until they were a heavy confluent layer and harvested by scraping with a rubber policeman. Cells which were converted by MSV were maintained as a continuous culture. The cells were harvested by scraping and by centrifuging detached and floating cells from the culture medium.

Isolation of plasma membranes

The BRL and virus-converted cells were suspended in 10 ml of 0.16 M NaCl per 36 (150-mm diameter) Falcon plates and homogenized with a Potter–Elvehjem homogenizer until all the cells were broken up. The homogenate was centrifuged at 200000 × *g* for 20 min and the resulting pellet suspended in 85 % (w/v) sucrose and brought to a refractive index of 1.430. Over the suspension was layered a linear 40–65 % (w/v) sucrose in water gradient (density = 1.150–1.241; total volume = 11 ml) and 1 ml of 10 % (w/v) sucrose. The material was centrifuged in a SW-41 rotor

of a Spinco-L2-65B ultracentrifuge at 90000 × *g* for 16.5 h. The discrete bands were removed from the gradient, 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 1.40 and overlaying them with a linear 15–45 % (w/v) sucrose gradient (density = 1.056–1.170) and 1 ml of 10 % (w/v) sucrose. The tubes were centrifuged at 90000 × *g* for 16.5 h, the bands removed, washed with saline or water, and saved for enzymatic or chemical analysis. In all experiments the quantities of enzymes, phospholipid, RNA, *etc.*, put on the gradient *versus* their distribution in the numerous fractions have been followed. The term particulate homogenate refers to the material obtained by homogenizing the cells in saline and sedimenting this homogenate at 200000 × *g*, resuspending the pellet in sucrose, and washing it with saline or water. Under these conditions most of the soluble proteins are removed.

Chemical procedures

The methods for determining protein, DNA, RNA, phospholipid, cholesterol, sialic acid, and neutral sugar expressed as glucose equivalents were the same as given in a previously published study[1]. Amino sugars were determined by the Elson–Morgan procedure as modified by ALLISON AND SMITH,[6] using glucosamine as a standard. The procedure of ALLISON AND SMITH, when half volumes were used for reagents and sample aliquots, showed no change in molar extinction coefficient. Aliquots for assay were purged with N_2, sealed, and hydrolyzed for 4 h in 4 M HCl at 100°. The hydrolysates were dried over NaOH in vacuum and then were dissolved in deionized water. These hydrolysis conditions gave maximum release of amino sugars with minimum destruction of freed amino sugars.

Enzyme assays

The activities of nucleotide phosphohydrolases, succinate dehydrogenase–coenzyme Q reductase (EC 1.3.99.1), and the NADH–cytochrome *c* reductase (EC 1.6.2.1) of the particulate control and gradient fractions were determined by methods given previously[1].

Light and electron microscopy

Intact cells grown on carbon slides were fixed and processed for light and electron microscopy according to the previously published method[2]. The cellular fractions were prepared for examination with the electron microscope by similar fixation, dehydration, embedding, and sectioning procedures. The sectioned material was examined in the Hitachi HU-11 B or C electron microscope at 75 kV accelerating voltage and at instrument magnifications of 1000–40000.

RESULTS

Light and electron microscopic observations of control and virus-converted cells

Cultured BRL cells grew as a monolayer with the cytoplasm flattened and spread out (Fig. 1). However, certain cells grew over those in the monolayer and formed thickened ridges, which tended to enclose the epithelial cells. These cells

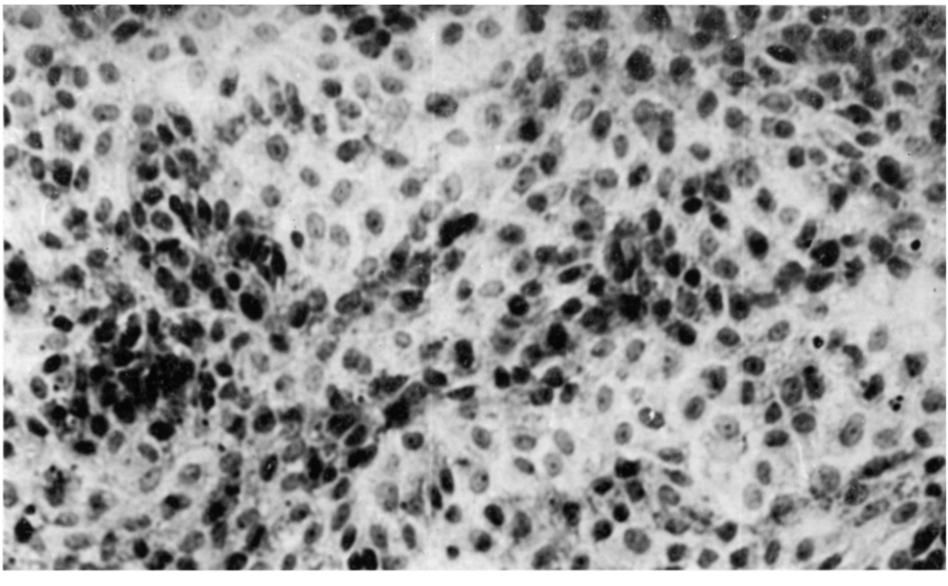

Fig. 1. A photomicrograph of parenchyma-like BRL cells which have been cultured to a high density. At high population densities, there was some overgrowth of the monolayer by round-shaped cells. × 75.

were round in shape. Electron microscopy of the BRL cells confirmed their epithelial character (Fig. 2). Intercellular junctions, some of them thickened, were established between cells, and there were small extensions of the plasma membrane into the intercellular space.

The cultured cells grew with a protein doubling time (from 2 mg per plate to 4 mg per plate) of 32 h. After implantation of 10^6 cells into Buffalo strain rats, no tumors arose in 12 months*.

The BRL cells which have been converted with MSV by TEMIN[4] are either fibroblast in shape with numerous pseudopodia or are spherical with cells piled one upon another (Fig. 3). The spherical-shaped cells frequently clumped together and floated in the culture medium. These floating cells were viable, as indicated by lack of staining with Trypan Blue and by reculturing them in fresh medium. The surface was irregular in the virus-converted, round-shaped cells (Figs. 4A and 4B). They have microvilli-like extensions of the plasma membrane, as was observed in the parent cells, but their cytoplasm also bulged out into lobes. These lobed surfaces formed intercellular junctions, some of which were of considerable length. The endoplasmic reticulum was less organized in the virus-converted cells, but the mitochondria remained unchanged. Virus particles were observed in the medium and between cells (Fig. 4B).

* The tumor-promoting properties of the BRL and MSV-converted cells were determined by Robert Reynolds. The BRL cells were placed in the abdominal cavity of male Buffalo rats in March, 1970, with no indication of tumors after 12 months. The MSV-converted cells, $0.5 \cdot 10^6$ and $1 \cdot 10^6$ cells, were injected subcutaneously over the shoulders of 1-day-old Sprague–Dawley rats and produced massive tumors by 2 weeks. Tumors also developed in animals which had received ultraviolet-killed cells; this indicates that oncogenic viruses were produced by these cells.

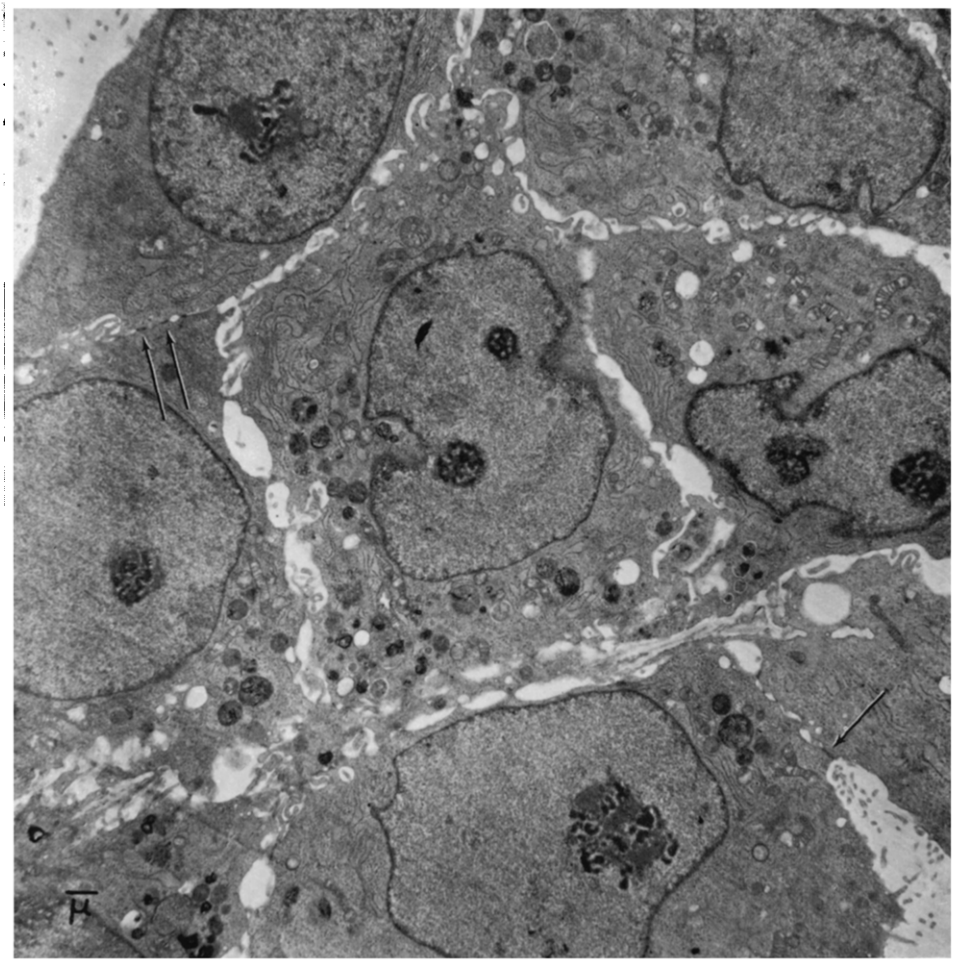

Fig. 2. A photoelectronmicrograph of a thin section from BRL cells which have been embedded while attached to a glass slide. These cells were arranged in an epithelial pattern with a moderate amount of extracellular space. This space was occluded in denser cultures. Cell membrane processes extended into the space between the cells and, when junctional complexes were formed, the cell membrane became thickened (arrows). Mitochondria and endoplasmic reticulum appeared to be normal in morphology and quantity. × 4000.

Injection of virus-converted and virus-producing BRL cells into 1-day-old rats produced massive tumors by 14 days*.

The separation of cellular components

Flotation equilibrium centrifugation of the material present in the particulate homogenate from both BRL and virus-converted cells resulted in the banding pattern shown in Fig. 5. This pattern was similar to that observed for gradient centrifugation of homogenates of chick embryo fibroblasts[1,2]. In this earlier work, the bands designated as A and B contained plasma membrane and were further purified by

* See footnote on p. 438.

centrifugation on a second continuous density gradient of sucrose. When the material present in the A and B regions of the gradient from the BRL cells was recentrifuged, the A′ and B′ distribution was achieved. In contrast to the gradients with material from chick fibroblasts, there was considerable overlap of these two bands, and material present in the upper one-third of the B′ gradient was combined with the membrane in the same region of the recentrifuged gradient of Band A. The mean density of a broad band of A′ was 1.081 ± 0.011 as determined by refractive index measure-

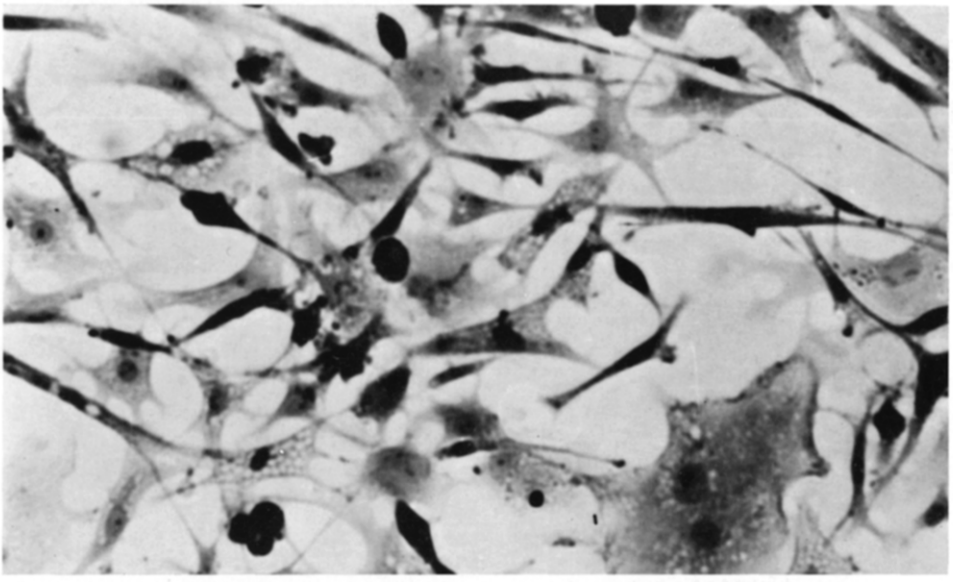

Fig. 3. A photomicrograph of BRL cells which have been converted by MSV. The virus-converted cells grew on the substratum with a shape and cell association pattern which was fibroblastic rather than epithelial. Round cells were observed in the culture. These cells frequently clumped together, detached from the culture dish, and were observed floating in the medium. × 75.

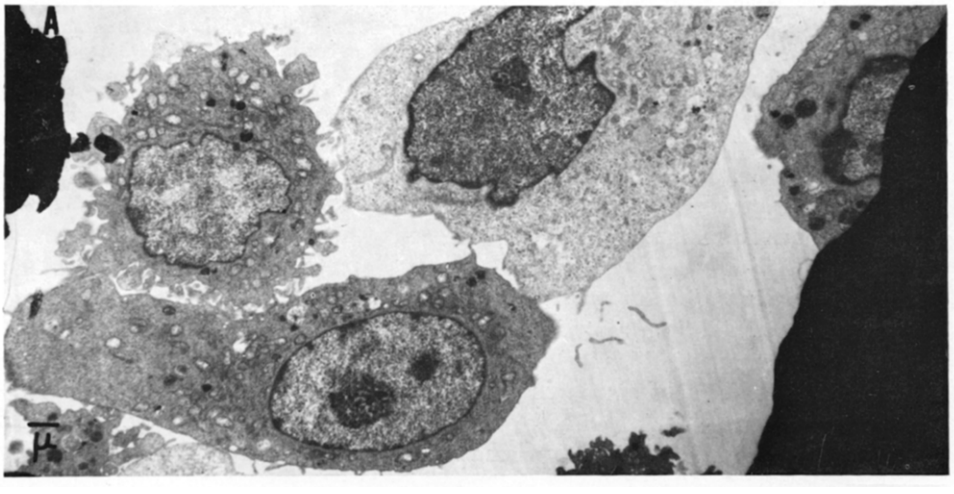

Fig. 4A.

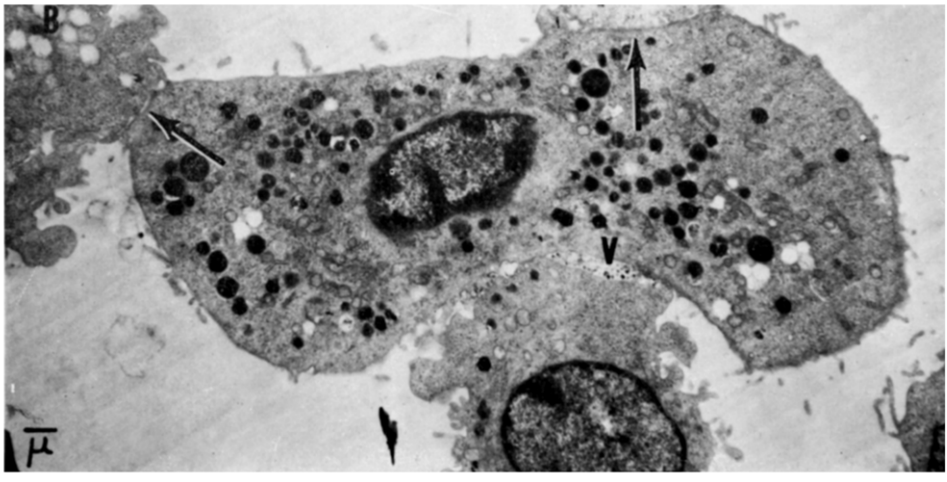

Fig. 4. A and B. Photoelectronmicrographs of thin sections of MSV-converted cells. Cell-to-cell interactions were observed less frequently in the virus-converted BRL cells than had been observed in the control cells. Converted cells which were round had very irregular surfaces. Their cytoplasm bulged out into lobes, which frequently formed the sites of intercellular junctions (4B, arrows). The fibroblast shaped cells had smoother surfaces, although fine villi-like processes were present on these cells as well as on the more highly lobed ones. Virus particles (V) accumulated in the culture medium and between cells. × 4000.

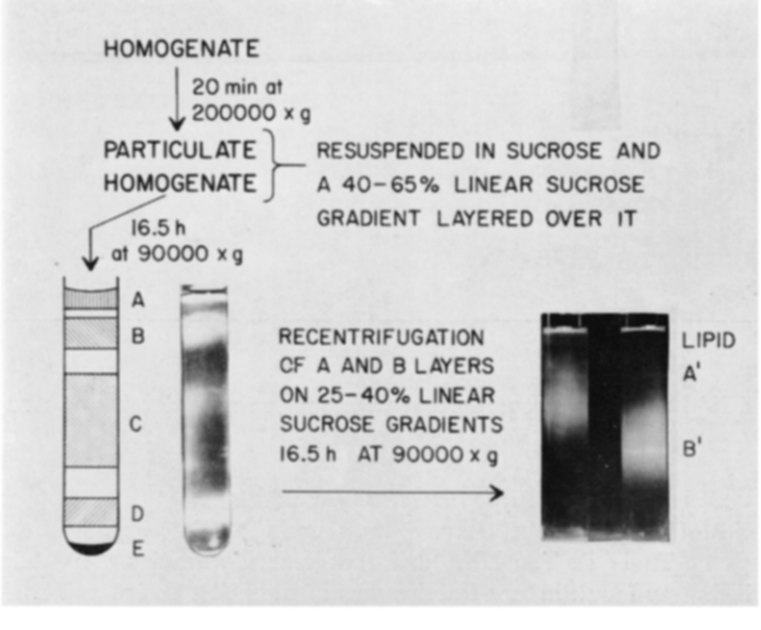

Fig. 5. Procedures used to isolate plasma membrane from BRL cells and cells converted by MSV. The banding pattern, with letter designations A–E, is presented both as a sketch and as a photograph of a gradient.

TABLE I

THE DISTRIBUTION OF MEMBRANE-ASSOCIATED ENZYMES AND CHEMICAL COMPONENTS AMONG BANDS A, CONVERTED BY MSV ON CONTINUOUS DENSITY GRADIENTS OF SUCROSE

The percent distribution of the membrane components present in the homogenate (100 %). The calculated the AMPase data, which were obtained from 4 experiments.

Component or enzyme		*Homogenate (units/mg protein)*	*Band A*	
			Units/mg protein	%
AMPase	BRL cells	3.41 ± 0.53	34.24 ± 7.72	3 ± 1
(μmoles P_i released/30 min)	MSV-converted cells	12.43 ± 0.75	75.97 ± 13.60	5 ± 2
CTPase	BRL cells	1.53 ± 0.25	9.23 ± 1.66	16 ± 4
(μmoles P_i released/30 min)	MSV-converted cells	0.55 ± 0.07	1.70 ± 0.49	3 ± 1
Cytochrome *c* reductase	BRL cells	0.112 ± 0.022	0.063 ± 0.010	2 ± 1
(μmoles reduced/min)	MSV-converted cells	0.144 ± 0.029	0.166 ± 0.057	2 ± 1
Succinate dehydrogenase	BRL cells	0.015 ± 0.002	0.001 ± 0.001	1
(μmoles indophenol reduced/min)	MSV-converted cells	0.012 ± 0.004	0.001 ± 0.001	1
Sialic acid (nmoles)	BRL cells	14 ± 1	71 ± 13	14 ± 4
	MSV-converted cells	14 ± 1	65 ± 6	8 ± 2
Glucose equivalents (μg)	BRL cells	124 ± 15	221 ± 26	5 ± 1
	MSV-converted cells	109 ± 4	217 ± 23	3 ± 1
Glucosamine equivalents (μg)	BRL cells	9.2 ± 0.7	24.0 ± 8.5	5 ± 2
	MSV-converted cells	9.1 ± 0.5	21.6 ± 1.0	3 ± 1
Phospholipid (μg)	BRL cells	213 ± 14	1080 ± 104	11 ± 3
	MSV-converted cells	227 ± 16	974 ± 103	8 ± 3
Cholesterol (μg)	BRL cells	56 ± 5	444 ± 66	16 ± 4
	MSV-converted cells	65 ± 4	399 ± 27	8 ± 2
RNA (μg)	BRL cells	60 ± 6	22 ± 15	1
	MSV-converted cells	103 ± 6	16 ± 10	1
DNA (μg)	BRL cells	131 ± 28	6 ± 1	0.2
Protein (mg)	BRL cells		2.4* ± 0.7	3 ± 1
	MSV-converted cells		1.2 ± 0.4	1 ± 1

* These values represent the total recovery of protein in the fraction.

ments. The broad B′ band was in equilibrium with sucrose at a density of 1.127 ± 0.005.

The biochemistry and morphology of fractions obtained from BRL cells

The recoveries of protein, DNA, enzyme, *etc.* among the Fractions A–E was between 60 and 75 % (Table I). The DNA and the greatest quantity of protein, RNA, and reducing sugar found in any fraction was in the pellet designated as E The enzymes, NADH–cytochrome *c* reductase and succinate–coenzyme Q reductase, considered as marker enzymes for endoplasmic reticulum and mitochondria, respectively, were concentrated in Bands B and C. Chemical components which were as-

B, C, D, AND A PELLET, E, FOLLOWING CENTRIFUGATION OF HOMOGENATES OF BRL CELLS AND CELLS

means ± S E. are based upon the combined results of from 5 to 9 experiments, with the exception of

Band B		*Band C*		*Band D*		*Pellet E*		*Recovery (%)*
Units/mg protein	%	*Units/mg protein*	%	*Units/mg protein*	%	*Units/mg protein*	%	
20.00 ± 3.47	42 ± 1	5.14 ± 1.37	17 ± 2	2.78 ± 1.07	18 ± 4	2.15 ± 0.82	16 ± 2	96
49.60 ± 3.30	40 ± 5	9.47 ± 0.82	15 ± 1	4.18 ± 0.20	11 ± 2	3.63 ± 0.45	15 ± 2	86
4.94 ± 1.89	21 ± 4	2.01 ± 0.30	10 ± 2	1.18 ± 0.31	6 ± 1	0.84 ± 0.20	16 ± 3	69
1.30 ± 0.16	23 ± 4	0.72 ± 0.12	16 ± 3	0.17 ± 0.03	7 ± 1	0.21 ± 0.03	12 ± 2	61
0.161 ± 0.047	11 ± 3	0.148 ± 0.042	21 ± 3	0.080 ± 0.014	12 ± 3	0.049 ± 0.010	13 ± 4	59
0.319 ± 0.084	18 ± 5	0.320 ± 0.064	22 ± 3	0.063 ± 0.016	9 ± 1	0.076 ± 0.017	11 ± 2	63
0.048 ± 0.010	41 ± 11	0.028 ± 0.014	22 ± 6	0.013 ± 0.010	7 ± 2	0.001 ± 0.001	4 ± 1	75
0.014 ± 0.004	18 ± 4	0.062 ± 0.023	32 ± 3	0.004 ± 0.002	7 ± 2	0.005 ± 0.003	6 ± 2	63
44 ± 9	21 ± 4	15 ± 2	11 ± 1	8 ± 1	8 ± 2	4 ± 1	13 ± 4	67
48 ± 7	30 ± 3	13 ± 2	13 ± 3	6 ± 2	10 ± 1	3 ± 1	5 ± 2	66
145 ± 32	8 ± 1	117 ± 11	10 ± 2	83 ± 4	19 ± 4	97 ± 1	34 ± 0	76
111 ± 11	10 ± 1	80 ± 9	13 ± 3	96 ± 8	19 ± 2	122 ± 5	29 ± 2	74
22.3 ± 1.6	18 ± 7	10.6 ± 0.8	13 ± 2	6.9 ± 0.7	16 ± 3	4.6 ± 0.1	20 ± 1	72
21.0 ± 3.6	23 ± 3	9.8 ± 1.7	17 ± 3	7.5 ± 0.5	17 ± 4	4.6 ± 0.1	22 ± 3	82
776 ± 160	19 ± 2	356 ± 36	9 ± 2	208 ± 35	9 ± 2	155 ± 26	24 ± 3	72
514 ± 73	21 ± 3	232 ± 17	18 ± 2	146 ± 22	12 ± 1	129 ± 19	15 ± 2	74
233 ± 38	22 ± 4	81 ± 7	11 ± 1	41 ± 8	7 ± 1	34 ± 7	14 ± 3	70
220 ± 26	30 ± 3	74 ± 9	13 ± 2	34 ± 2	11 ± 1	32 ± 3	13 ± 3	75
28 ± 6	3 ± 1	43 ± 5	9 ± 3	60 ± 8	16 ± 3	57 ± 11	38 ± 8	67
42 ± 6	5 ± 2	66 ± 3	11 ± 3	94 ± 4	23 ± 2	93 ± 5	24 ± 4	64
11 ± 7	0.5	22 ± 7	1 ± 1	57 ± 22	12 ± 1	241 ± 13	62 ± 1	75
6.6* ± 1.3	8 ± 2	7.5* ± 2.2	11 ± 2	9.8* ± 2.3	14 ± 3	28.6* ± 6.5	41 ± 6	77
10.3 ± 2.0	10 ± 2	13.0 ± 2.0	13 ± 3	25.9 ± 5.0	22 ± 3	30.4 ± 6.1	25 ± 3	71

sociated with the isolated plasma membrane from chick fibroblasts[1,2], namely sialic acid, phospholipid, cholesterol, and CTPase activity, were concentrated within the upper layers of the gradient, in bands A and B. These components were also in the A band from the BRL cells. The membranes within this layer accounted for 3 % of the protein put on the gradient and 11–16 % of the sialic acid, lipid, and CTPase activity relative to the particulate homogenate as 100 %. The distribution of AMPase activity among the layers on the gradient also established this enzyme as a marker for the cell membrane. The highest specific activity was found in the A band, and this fraction accounted for 3–10 % of the units of this enzyme present in the homogenate. This layer was contaminated by a considerable amount of microsome-associated

enzyme and a few mitochondria. Recentrifugation of the material present in the A band removed mitochondria and decreased the specific activity of the microsome-associated enzyme and RNA (Table II). Recentrifugation of the material present in the B band resulted in similar decreases in the amount of mitochondrial and microsome-associated NADH–cytochrome *c* reductase found in the B′ layer (Table II). The membranes present in the B and B′ layer, although contaminated with intracellular membranes and organelles, have specific concentrations of sialic acid, AMPase and CTPase activity, phospholipid, and cholesterol which are 2.5 times greater than those of the particulate homogenate, which findings indicate that these membranes are plasma membranes. The distribution of neutral sugars and amino sugars among

TABLE II

THE DISTRIBUTION OF MEMBRANE-ASSOCIATED ENZYMES AND CHEMICAL COMPONENTS BETWEEN BANDS A′ AND B′ FOLLOWING THE RECENTRIFUGATION OF THE A AND B BANDS FROM HOMOGENATES OF BRL CELLS AND CELLS CONVERTED BY MSV ON CONTINUOUS DENSITY GRADIENTS OF SUCROSE

The percent distribution of the membrane components present in the A band (100%) and the B band (100%). The calculated means ± S.E. are based on the results of 5–9 experiments with the exception of the AMPase data which was obtained with 4 experiments.

Components or enzymes		*Band A′*		*Band B′*	
		Units/mg protein	%	*Units/mg protein*	%
AMPase	BRL cells	50.29 ± 13.70	65 ± 10	31.45 ± 5.91	38 ± 7
(μmoles P_i released/30 min)	MSV-converted cells	88.71 ± 14.10	60 ± 11	67.65 ± 13.70	45 ± 9
CTPase	BRL cells	7.52 ± 2.09	61 ± 16	4.03 ± 1.36	28 ± 6
(μmoles P_i released/30 min)	MSV-converted cells	1.09 ± 0.29	84 ± 27	1.30 ± 0.32	39 ± 6
Cytochrome *c* reductase	BRL cells	0.034 ± 0.010	44 ± 17	0.059 ± 0.024	19 ± 8
(μmoles reduced/min)	MSV-converted cells	0.092 ± 0.040	35 ± 14	0.182 ± 0.040	24 ± 4
Succinic dehydrogenase	BRL cells	0.00	0	0.002 ± 0.001	8 ± 7
(μmoles indophenol reduced/min)	MSV-converted cells	0.00	0	0.002 ± 0.001	6 ± 4
Sialic acid (nmoles)	BRL cells	68 ± 17	59 ± 16	52 ± 6	38 ± 6
	MSV-converted cells	63 ± 7	96 ± 30	41 ± 5	37 ± 4
Glucose equivalents (μg)	BRL cells	300 ± 35	73 ± 18	167 ± 19	43 ± 5
	MSV-converted cells	367 ± 42	89 ± 15	122 ± 19	48 ± 7
Glucosamine equivalents (μg)	BRL cells	29.2 ± 6.5	55 ± 18	21.7 ± 0.9	40 ± 13
	MSV-converted cells	26.1 ± 10.3	90 ± 17	22.5 ± 5.8	41 ± 10
Phospholipid (μg)	BRL cells	1012 ± 134	51 ± 10	595 ± 126	34 ± 7
	MSV-converted cells	1079 ± 131	71 ± 15	507 ± 83	44 ± 7
Cholesterol (μg)	BRL cells	489 ± 87	58 ± 13	205 ± 39	33 ± 5
	MSV-converted cells	471 ± 65	81 ± 13	200 ± 23	38 ± 4
RNA (μg)	BRL cells	12 ± 8	15 ± 9	21 ± 2	31 ± 5
	MSV-converted cells	9 ± 6	9 ± 9	40 ± 6	43 ± 8
Protein (mg)	BRL cells	0.81* ± 0.25	55 ± 14	1.24* ± 0.34	40 ± 7
	MSV-converted cells	0.67 ± 0.19	98 ± 23	2.32 ± 0.45	43 ± 5

* These values represent the total recovery of protein in the fraction.

the gradient fractions was unequal, with the highest specific concentrations found among the membranes present in the A and B layers. The amino sugar distribution was particularly unique, since the sialic acid and neutral sugar were concentrated in

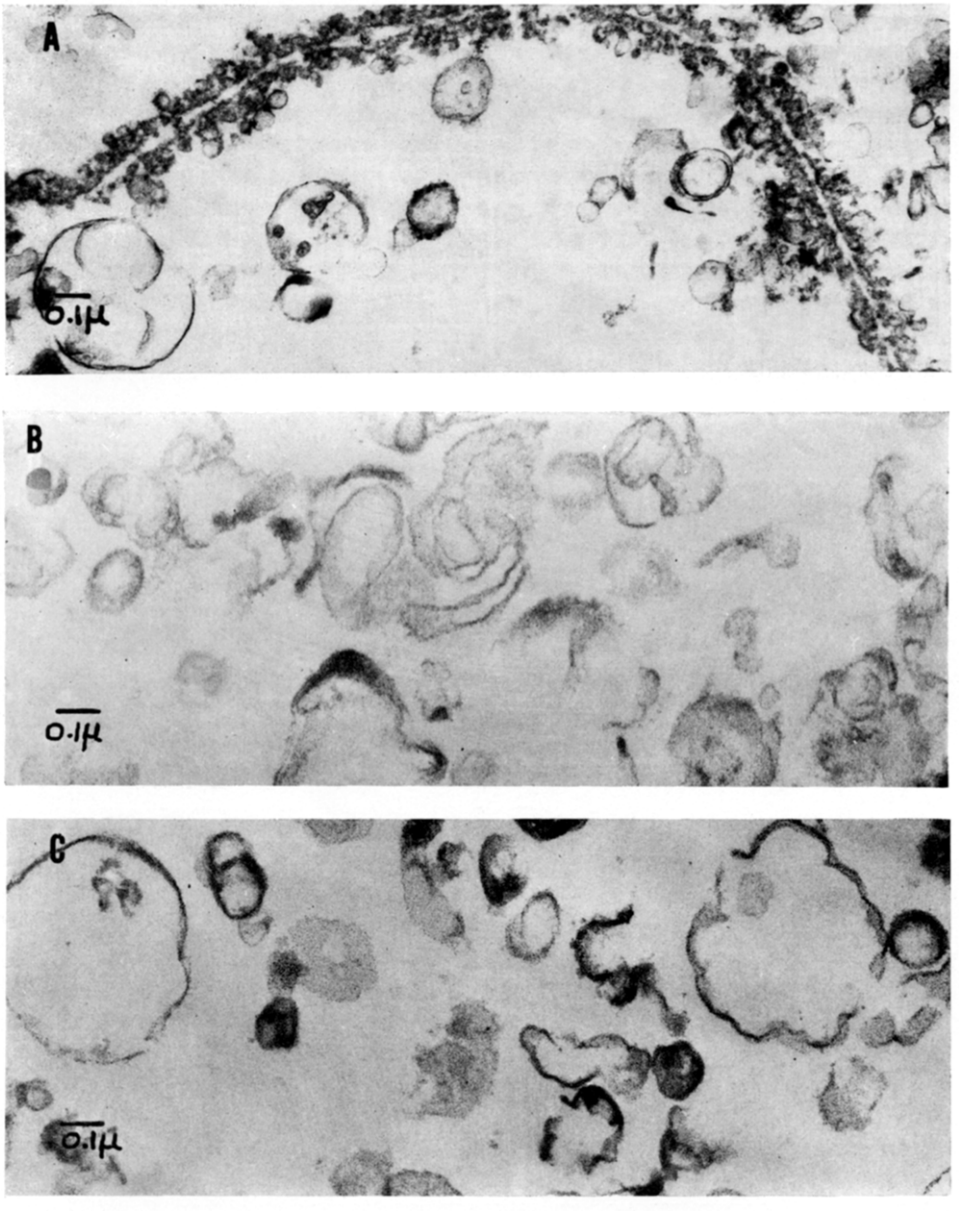

Fig. 6. A, B and C. Photoelectronmicrographs of thin sections of membrane in the A′ band from BRL cells (A and B) and cells converted by virus (C). The membranes from both preparations were isolated in the form of closed vesicles. Occasionally, junctional complexes with vesicles attached to their margin have been observed in preparations from the BRL cells (A), but never in membrane preparations from virus-converted cells. A, × 36000; B and C, × 55000.

membranes from the A and A′ bands while it was approximately the same concentration in the membranes from the B and B′ bands.

The membrane fragments present in the A and A′ bands frequently form small, closed vesicles (Fig. 6B). Occasionally, long stretches of membrane with vesicles

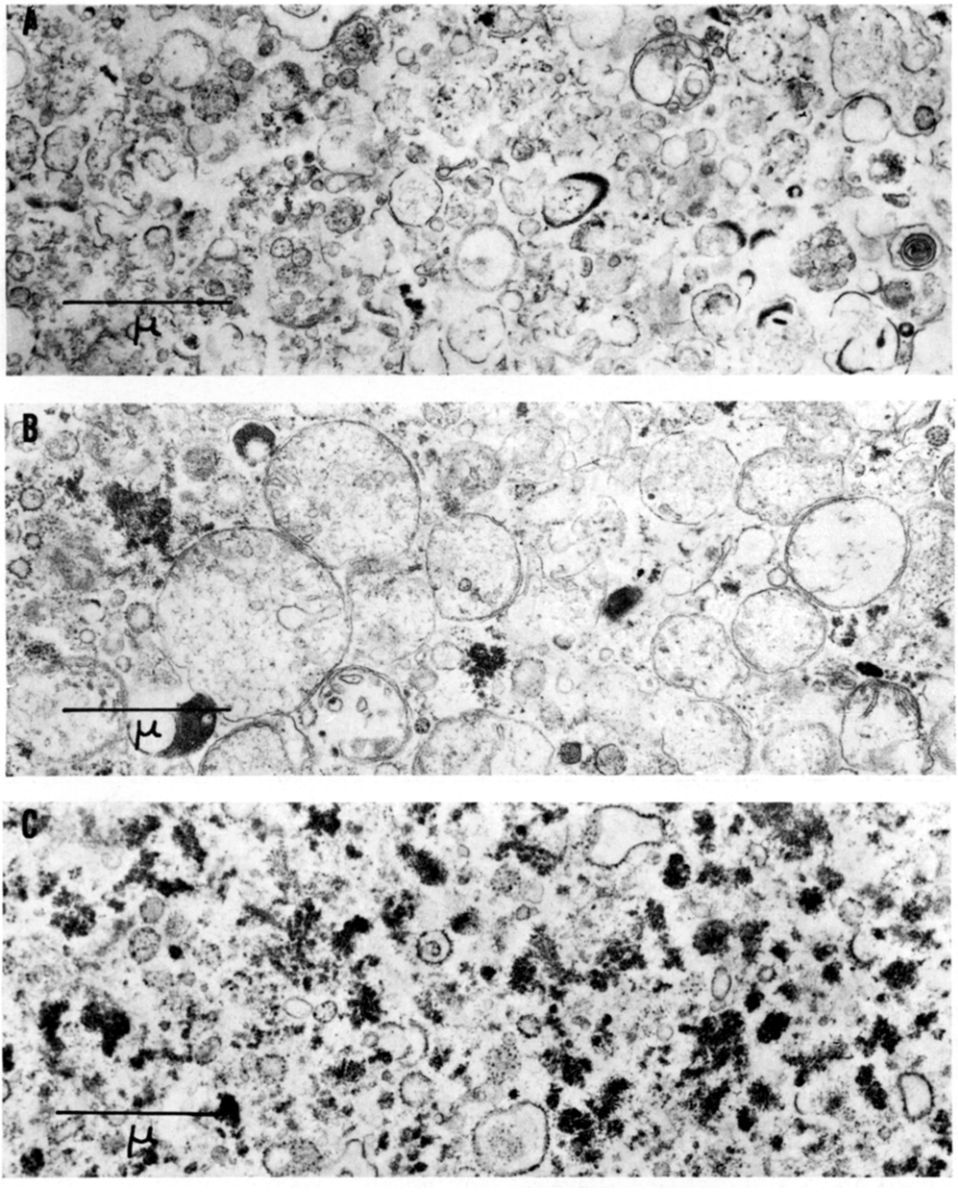

Fig. 7. Photoelectronmicrographs of thin sections prepared from pellets of the B, C, and D bands. A. The B band contained smooth and rough endoplasmic reticulum, presumably plasma membrane, and mitochondria. These latter organelles were removed by centrifugation on the second sucrose gradient. B. Mitochondria were particularly numerous in the C band. This fraction also contained smooth and rough reticulum and nucleolar-like material. C. The RNA-containing reticulum and nucleolar material were concentrated in the D layer. × 22000.

attached to their margins, have been observed (Fig. 6A), as well as junctional complexes holding adjacent cells together, similar to results reported for liver[7]. The B and B′ fractions contain membrane fragments, the longer pieces of which may be plasma membrane or may have arisen from swollen mitochondria. Mitochondria are present in large numbers in the B layer, but are not found in the B′ band (Fig. 7A). Small vesicles, most of them free of ribosomes, are the predominant membranes found in this fraction. The C band was composed of many mitochondria, rough endoplasmic reticulum, some smooth membranes, and nucleolar-like dense material (Fig. 7B). This presumably RNA-rich nucleolar material was concentrated in the D band along with ribosome-associated membranes (Fig. 7C). The cellular components present in the pellet, the E band, were mainly broken nuclei, nucleolar material, a few mitochondria, and smooth and ribosome-associated membranes.

Biochemistry of fractions from the gradient of virus-converted cells

The equilibrium centrifugation banding pattern for the homogenates of MSV-converted cells was similar to that observed for BRL cells (Fig. 5). The recoveries of components put on the gradient were also similar, although there was some shifting with fractions. Only 1 % of the original protein was recovered in the A band, but there was a 2 % increase of protein within the B band (Table I). This difference

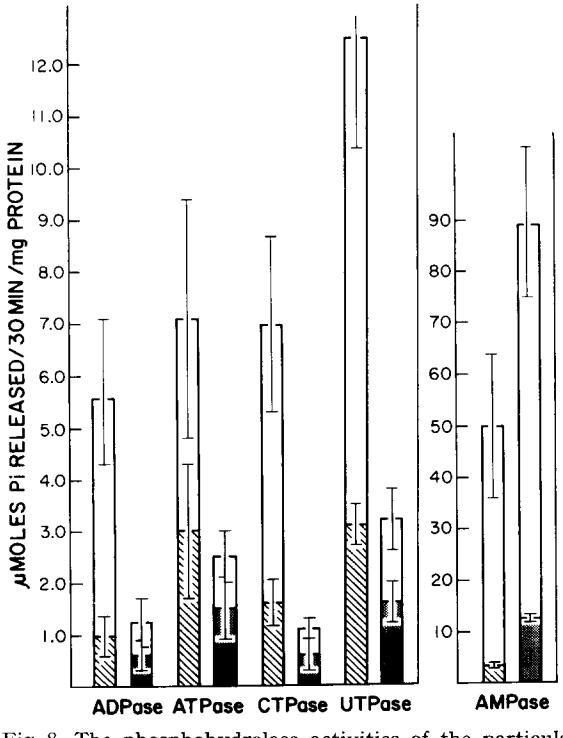

Fig. 8. The phosphohydrolase activities of the particulate homogenate and membranes in the A band from BRL cells and MSV-converted BRL cells. Membrane fractions from the particulate homogenate and A band from BRL cells (▨, □) and from MSV-converted cells (▨, □), respectively, were incubated at 37° in 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 calculated means ± S.E. are based on the results of five experiments.

represented material of A-band composition which remained with the B band, since recentrifugation of the B band resulted in a large quantity of membrane moving into a position on the second gradient occupied by A′-band membrane. There was also some shift in the recoveries of components within the D band and the pellet, the E band.

The percent distribution, specific concentrations, and enzymatic activities of the cellular components present in the MSV-converted cells were similar to those found for the BRL cells. The quantities of sialic acid, phospholipid, and cholesterol in the membranes of the A and A′ bands were not significantly different for the two preparations (Tables I and II). However, the specific activity of the NADH–cytochrome *c* reductase that was found with both the A′ and B′ membranes was 3 times greater for the MSV-converted cells (Table II). An even more striking difference was observed for the specific activity of CTPase. In the particulate homogenate from the virus-converted cells, the activity of this enzyme was one-third that of the BRL cells. The CTPase activity associated with the plasma membrane found in the A and the A′ layers from the MSV-converted cells was one-fifth to one-sixth that of the comparable isolated membranes from the uninfected cells. The AMPase activity of the virus-converted cell membrane was increased (Tables I and II), but the enzymatic activities of ADPase, ATPase, and UTPase were reduced significantly (Fig. 8). No other chemical differences were as obvious as those of the nucleotide phosphohydrolases. The neutral sugar content of the A′-band membrane increased slightly in the virus-converted cells (Table II) but, because of the variability between determinations, this difference was not significant. The amino sugar content of the plasma membrane from these cells was like that of the uninfected cells. Thin sections of the material present in bands from the virus-converted cells indicated the same distribution of components as was found with the gradient from the BRL cells. The membranes in the A′ bands were small and vesicular (Fig. 6C) and indistinguishable from plasma membrane prepared from the BRL cells.

DISCUSSION

The method of flotation equilibrium centrifugation of a homogenate of cultured liver cells (BRL-3A) resulted in the preparation of plasma membranes which were similar in properties to those obtained from cultured chick embryo fibroblasts[1,2]. The BRL cell membranes had a density in sucrose of 1.08 a value identical to that found for fibroblast cell membranes[1], and the same chemical components were concentrated in these membranes as had been found to be concentrated in the fibroblast membrane (*i.e.*, sialic acid, phospholipid, cholesterol, neutral sugar, and CTPase). However, the specific concentrations, expressed as μg per mg protein, and the calculated composition of the cell membrane (Table III) were not the same. The phospholipid and cholesterol content of the BRL cell membrane was about 60 % of that found for the fibroblast membrane. Both cell membrane preparations were obtained free of mitochondria. However, the amount of NADH–cytochrome *c* reductase was 3 times higher in the A′ band from parenchymal cells than had been found in the fibroblasts. The specific activity of the starting material was also 3 times higher, and it has been reported by VASSILETZ *et al.*[8] that these enzymes are components of the plasma membrane from rat liver. The carbohydrate content and the CTPase activity of these two preparations were similar.

TABLE III

THE CALCULATED COMPOSITION OF THE MEMBRANE PRESENT IN THE A′ BANDS FROM BRL AND MSV-CONVERTED CELLS

The concentration of all the components, expressed as μg/mg protein, as shown in Table II, was 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	*BRL cells A′* (%)	*MSV-converted cells A′* (%)
Protein	35.0	33.7
Phospholipid	35.5	36.4
Cholesterol	16.8	15.6
Sialic acid	0.7	0.6
Carbohydrate in glucose equivalents	10.3	12.2
Amino sugar in glucosamine equivalents	1.0	0.9
RNA	0.4	0.2

Modifications of the method of NEVILLE[9] have been used most frequently to isolate plasma membrane from liver. This procedure employs centrifugation of homogenates on discontinuous density gradients of sucrose with the accumulation of membrane between the interfaces. The yields of plasma membrane and the amount of contamination with intracellular membranes and organelles varied among the different preparations[7–14], making it difficult to compare the properties of the isolated plasma membrane from the BRL cells with that isolated from the whole organ. The 1:1 ratio of phospholipid to protein found for the cultured cells was much higher than has been reported for most plasma membrane preparations[15]. RAY[14], COLEMAN *et al.*[12], and DOD AND GRAY[16] found about 600 μg of phospholipid per mg protein, but other investigators have reported lower values. An exception to these findings and one which is in agreement with our results was recently reported by EVANS[17]. He observed that the plasma membrane from mouse liver, isolated by zonal centrifugation procedures at a density of 1.17, could be separated by isopycnic centrifugation into two cell membrane fractions, one at a density of 1.12 and the other at 1.18. The chemical and enzymatic characterization of these fractions established them both as plasma membrane, but the distribution of these components between the light and heavy fractions was distinctive. The membrane of density 1.12 had 1600 μg phospholipid per mg protein, whereas the membrane of density 1.18 had 850 μg per mg protein. The mouse liver cell membrane isolated by zonal centrifugation had 950 μg phospholipid per mg protein; the BRL cell plasma membrane contained 1012 μg per mg protein.

The cholesterol content of the plasma membrane isolated by EVANS[17] was 200 μg per g protein; this was about half of the amount of cholesterol found in the cell membrane from the cultured cells. It had been reported by COLEMAN AND FINEAN[18] that, as the plasma membrane from liver was purified, the ratio of cholesterol to phospholipid increased. This observation had been confirmed by BOSMANN *et al.*[19] for HeLa cells. An increasing ratio of cholesterol to phospholipid was found for the material in Bands E–A from both the BRL cells and the virus-converted ones (Table IV). The less dense the membrane or particulate fraction, the greater was its cholesterol/phospholipid ratio. A ratio of 0.9 was found for the plasma membrane from

TABLE IV

THE CHOLESTEROL/PHOSPHOLIPID MOLAR RATIOS OF BRL AND MSV-CONVERTED CELL FRACTIONS

The cholesterol and phospholipid values in Tables I and II were converted to μmoles/mg protein, with molecular weight values of 386 and 775, respectively, used for the conversion.

Fraction	*Cholesterol/Phospholipid ratio*	
	BRL cells	*MSV-converted cells*
Homogenate	0.529	0.575
Band A	0.825	0.821
Band A′	0.970	0.876
Band B	0.602	0.858
Band B′	0.692	0.792
Band C	0.455	0.638
Band D	0.395	0.468
Pellet E	0.440	0.493

the cultured cells; ratios of 0.38–0.7 have been reported for cell membrane isolated from liver.

The sialic acid content of plasma membrane isolated from the cultured cells, 68 nmoles per mg protein, was higher than had been found by BENEDETTI AND EMMELOT[7,15] and by RAY[14] for plasma membrane isolated from liver by discontinuous density gradient centrifugation and by EVANS[17] for membrane isolated by zonal centrifugation. It was identical, however, to the latter author's subfractionated liver plasma membrane that had a density of 1.12.

The neutral sugar content of the isolated cell membrane from parenchyma-like cells was 10–20 times greater than had been reported for isolated liver cell membrane[15], but it had about the same number of glucose equivalents as plasma membrane isolated from the chick fibroblasts[1,2]. The equal distribution of amino sugar in the A, A′, B, and B′ bands was unexpected. The content of sialic acid and neutral sugar among the various particulate fractions had shown a constant progression from low levels in the lower bands on the gradient to the highest levels in the A′ band. It had been anticipated that amino sugar distributions would be similar. A possible explanation may be that some of the sialic acids in the A′ band are components of glycolipids. The glycolipids are rich in galactosamine, and this sugar has a lower molar extinction coefficient in the Elson–Morgan reaction than glucosamine, the standard for these reactions. To resolve this inconsistency, it will be necessary to determine the molar ratios of the amino sugars and neutral sugars; this is being studied.

Are the parenchyma-like cells still epithelial in character? These cells had their origin as a clone from cultures of young rat liver of the Buffalo strain. COON[5] has ascribed some functional properties to a cell line isolated in a similar fashion, but attempts to induce tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) by hydrocortisone and insulin have not been successful. The mean basal enzyme level for untreated cells was 3.7 ± 0.35 μmoles of *p*-hydroxyphenylpyruvate per mg cell protein per 30 min of incubation. BRL cells which were cultured for 16 h with inducing levels of hormones[20] had a specific activity of 2.6 ± 0.2. These cells still appeared to be epithelial in their shape but there were some cells

which no longer grew as a monolayer. In certain regions of the cultures round cells piled up, one upon the other. However, cells which were placed in Buffalo strain rats for 12 months have as yet not given rise to tumors. Therefore, with reservation, we have assumed that because of their epithelial-like shape and adhesive properties these cells served as adequate models for comparing their plasma membrane composition with that of membrane isolated from virus-converted cells.

The addition of MSV radically changed the morphology and adhesive properties of these BRL cells. The virus-converted cells assumed more elongated shapes and round, highly lobulated shapes in which cells were piled one upon the other. The cells formed junctions with each other, but the presence of these associations can not be taken as evidence that the adhesive nature of these cells was unchanged. The decreased surface tension, evidenced by the lobulated cell membrane extensions, permits the interaction of one cell with another along long stretches of cell surface. A similar observation was made for chick fibroblasts converted by RNA sarcoma producing viruses[2,21].

The virus-converted BRL cells provide us with a line of cells that produces tumors in rats*, either by themselves or through the production of oncogenic viruses, have an altered morphology, and have modified adhesive properties. Yet, membranes of these cells are very similar in chemical composition when compared with membranes of the cells of origin (Table III). The specific concentrations of lipid and carbohydrate are the same for the two cells. Although on a percent compositional basis there may be a small increase in neutral sugar, the amount of membrane-bound sialic acid, which decreased in virus-converted chick fibroblasts along with a concomitant increase in neutral sugar[2], remained constant. It must be restated that these measurements are unspecific in that they do not indicate the composition or the quantity of the sugars that are associated with these membranes. The membrane-associated enzyme activities in which changes occurred were those of CTPase, AMPase, ADPase, ATPase, and UTPase. The specific activity of the CTPase decreased from 7.52 to 1.09. The role of enzymes which hydrolyze these substrates remains unknown. The problem becomes even more puzzling in that the AMPase activity, which was already high in these cells, increased still further.

In conclusion, a cultured parenchyma-like cell from rat liver, BRL, can be converted by sarcoma-producing viruses into a cell which is greatly altered in its shape, its association with other cells, and its adhesion to the substrate. However, there have not been any major changes in cell membrane chemistry in the parameters we have examined other than the marked decrease and increase in the activity of nucleotide phosphohydrolases. It might have been anticipated that, if adhesion were the result of the presence of cell surface cement, that some chemical alteration would have been detected. In chick fibroblasts that have been converted by RNA sarcoma-producing viruses, modifications of shape and adhesion accompany qualitative and quantitative changes in cell membrane chemistry. One must consider, therefore, that either there is no one common property of all cells which regulates adhesion or, at the very least, adhesion is not manifested by the presence of some gross chemical constituent of the cell membrane but is a combination of intracytoplasmic organization and specialized cell surface chemistry.

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* See footnote on p. 438.

A detached cell will round up because of the tension present within its cell membrane or cortical regions[22]. To maintain a highly asymmetric form, as is the case with epithelial cells, intracytoplasmic forces must be generated. At another level, the summation of the processes of cell movement and the establishment, breakage, and reestablishment of intercellular adhesions produce form in developing organisms[22]. Recent studies by WESSELLS *et al.*[23] have implicated microfilaments, 50 Å in diameter, as essential components in morphogenic development; they suggested a contractile function for these cytoplasmic elements.

Cultured chick fibroblasts and the BRL cells studied in this laboratory contain microfilaments. JOHNSTON[21] studied the distribution of these microfilaments within chick fibroblasts and fibroblasts converted by RNA sarcoma virus and correlated a decrease in the cortical organization of these filaments with the change in shape of the virus-converted cell. Recently, YANG *et al.*[24] isolated a contractile protein from chick fibroblasts which has enzymatic, chemical, conformational and, morphogenic properties like those of rabbit actin.

Considering these findings, it is conceivable that the cell membrane, with its specialized chemistry and enzymes, regulates the responses of the cell to environmental stimuli including the presence of other cells and, in the case of cancer cells, determines their altered responses as well. But the cell-generated forces which respond to the instructions from the plasma membrane and which function in the maintenance of shape and adhesion to the substratum reside within organized cytoplasmic elements including microtubules and microfilaments. Perhaps it should be considered that the genetically expressed alteration in the chemistry, controlled assembly, or function of these cytoplasmic structures may hold the key to our understanding of the manifestations of malignancy, such as cellular invasion and metastasis.

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