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THE ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANE FROM CULTURED CELLS.

I. THE CHEMICAL COMPOSITION OF MEMBRANE ISOLATED FROM UNINFECTED AND ONCOGENIC RNA VIRUS-CONVERTED CHICK EMBRYO FIBROBLASTS

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SUMMARY

1. Plasma membrane was isolated from cultures of chick embryo fibroblasts and fibroblasts which had been infected with the avian sarcoma virus, RBA, and the avian leukosis virus, RAV-49.

2. On continuous density gradients of sucrose, the cell membrane was localized in two fractions, the A′ band and B′ band. The membranes in the A′ band contained twice as much phospholipid and cholesterol as the membranes in the B′ band. The membranes in the latter band also contained less neutral sugar and sialic acid.

3. Plasma membrane in the A′ band that was isolated from the oncogenic virus-converted cells, RBA, had increased levels of neutral sugar (358 *vs.* 243 μg per mg protein), but the sialic acid content of this membrane was decreased by 25 %.

4. Cell membrane obtained from the leukosis virus-infected cells had quantities of sialic acid and neutral sugar that were similar to those of uninfected cells. Therefore, the increase in neutral sugar and the decrease in sialic acid found in cell membrane from the RBA-converted cells are chemical modifications of the cell surface which reflect conversion by an oncogenic virus and are not the result of virus infection *per se*.

INTRODUCTION

Infection of cultured cells with oncogenic viruses results in alterations in the quantitative controls of cell multiplication[1]. Changes in the chemistry of the cell membrane, which may have a causative relationship to the decreased adhesiveness and subsequent invasiveness and metastasis of cancer cells, accompany altered cell growth[2]. These plasma membrane modifications are expressed in changed cellular adhesiveness[3], in loss of intercellular communication[4], in altered cell surface charge[5], and in the deletion or emergence of membrane components (lipids, proteins, or carbohydrates) which affect metabolite transport[6,7] and the antigenicity[8–11] and agglutinability[12–15] of cells.

A method has been developed for isolating cell membranes from cultured chick embryo fibroblasts[16]. The isolated plasma membrane fractions were of a high degree of purity and were concentrated in sialic acid, phospholipid, cholesterol, and nucleotide phosphohydrolases[16]. The methodology employed in this earlier work was used to isolate plasma membrane from cultured fibroblasts that had been converted[1] by the avian sarcoma virus, B_{77} (ref. 17). The chemical and enzymatic properties of this membrane were determined and compared with those from membranes isolated from genetically identical but infected cells. This study establishes that morphological conversion by B_{77} is correlated with a decrease in cell surface sialic acid and an increase in neutral sugars.

MATERIALS AND METHODS

Fertilized chicken eggs were supplied by Sunnyside Hatchery of Oregon, Wisc. Fertilized eggs from an inbred strain of white leghorn (RPL-15) chickens were kindly provided by W. H. McGibbon of the Department of Poultry Science, University of Wisconsin. Avian RNA sarcoma-producing virus, Bratislava 77 (ref. 17) which had been passed through rat cells and back again into chick embryo fibroblasts and designated RBA (ref. 18) and avian RNA leukosis virus, RAV-49 (ref. 19), were obtained from H. M. Temin. The modified minimal essential medium (Eagle's) was obtained from Schwarz-Mann; the calf serum, from Microbiological Associates; and the fetal calf serum, from Grand Island Biological Co. Collagenase was obtained from Worthington.

Cell culturing

Primary cultures of 12-day-old chick embryo fibroblasts were prepared by a method described by TEMIN[20] and were grown at 37° in a humidified CO_2 incubator in Eagle's modified essential medium containing 4% calf serum or 2% fetal calf serum. 5 days later, the fibroblasts were transferred to Falcon tissue culture dishes (150-mm diameter) at a concentration of $4 \cdot 10^6$ cells per plate. The medium used to culture the secondaries contained 6% calf serum or 3% fetal calf serum.

Infection of fibroblasts with virus

24 h after transfer, the secondaries from one-half of the primary were infected with $1.5 \cdot 10^7$–$3 \cdot 10^7$ focus-forming units of RBA or RAV-49 per plate. The conversion of the fibroblasts by these viruses was enhanced by adding 3 μg of DEAE-dextran to the culture at the time of infection[21]. The cells were cultured until they displayed a characteristic rounding up. This was evident by 5–10 days after infection with the RBA virus. Cells infected with RAV-49 maintained their fibroblast shape.

Harvesting of cells and the isolation of plasma membranes

Fibroblasts which were prepared from embryos from the Hatchery's eggs 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. Fibroblasts and cells converted by virus which were obtained from the inbred strain of chicken embryos were rinsed twice with 0.16 M NaCl and removed by scraping with a rubber policeman. The cells released by either of the two methods were suspended in 0.16 M NaCl, centrifuged, and washed twice by resuspension and centrifugation.

The washed cells were suspended in 10 ml of 0.16 M NaCl per 36 tissue culture 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 was resuspended in 85% (w/v) sucrose and brought to a refractive index of 1.430. A linear sucrose gradient was layered over the suspension and the material centrifuged according to the previously published method[16]. The layers A and B were removed and run on a second linear sucrose gradient[16]. The discrete bands of cellular components were removed, diluted with 0.16 M NaCl or water, centrifuged, and washed twice by resuspension, dilution with saline or water, and recentrifugation. The washed fractions were saved for enzymatic or chemical analyses. In all experiments, the quantity of enzymes, phospholipids, RNA, *etc.*, put on the gradient was compared with their distribution in the numerous fractions. 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 protein, DNA, RNA, phospholipid, cholesterol, sialic acid, and neutral sugar expressed as glucose equivalents were determined according to the previously described procedure[16].

Enzyme assays

The activities of nucleotide phosphohydrolase, succinate dehydrogenase–coenzyme Q reductase (EC 1.3.99.1), and the NADH–cytochrome *c* reductase (EC 1.6.2.1) were determined according to the previously described methods[16,22].

Light and electron microscopy

Intact cells grown on carbon-coated slides[23] were fixed for 1 h at 0–4° with 1% glutaraldehyde, 50 mM cacodylate buffer (pH 7.2), and 250 mM sucrose[24]. Certain of the cells were examined with Nomarski interference optics while others were stained with hematoxylin and eosin and examined with the light microscope. Cells on other slides were "post-fixed" in 1% osmium tetroxide, 50 mM cacodylate buffer (pH 7.2) and 250 mM sucrose for 1 h at 0–4° and dehydrated in a series of ethanol solutions of increasing concentrations. The microscope slide with adhering cells was drained, and an epoxy-embedding medium[25] was layered over it and left at room temperature for 3 h to permit infiltration of the cells. The slides were drained of medium and BEEM capsules containing embedding medium were placed over representative cells. The plastic was polymerized by incubation at 65°. Thin sections of the flat-embedded specimens were cut with diamond or glass knives, mounted on carbon-covered grids, and stained with lead citrate[26].

Preparations of cell membrane were examined in negative contrast by placing them on carbon-covered grids and fixing for 30 min at 4° in 1% glutaraldehyde containing 10 mM cacodylate buffer (pH 7.2), sucrose, and water to 250 mosM. The fixed material was rinsed with 10 mM cacodylate to remove sucrose and stained with 1% phosphotungstic acid (pH 7.2), according to the procedure of BRENNER AND HORNE[27]. In some cases the membranes were post-fixed with 1% osmium containing

buffer and sucrose prior to being stained with phosphotungstic acid. The sectioned and negatively stained material was examined in the Hitachi HU-11C electron microscope at 75 kV accelerating voltage and at instrument magnifications of between 1000 and 60000.

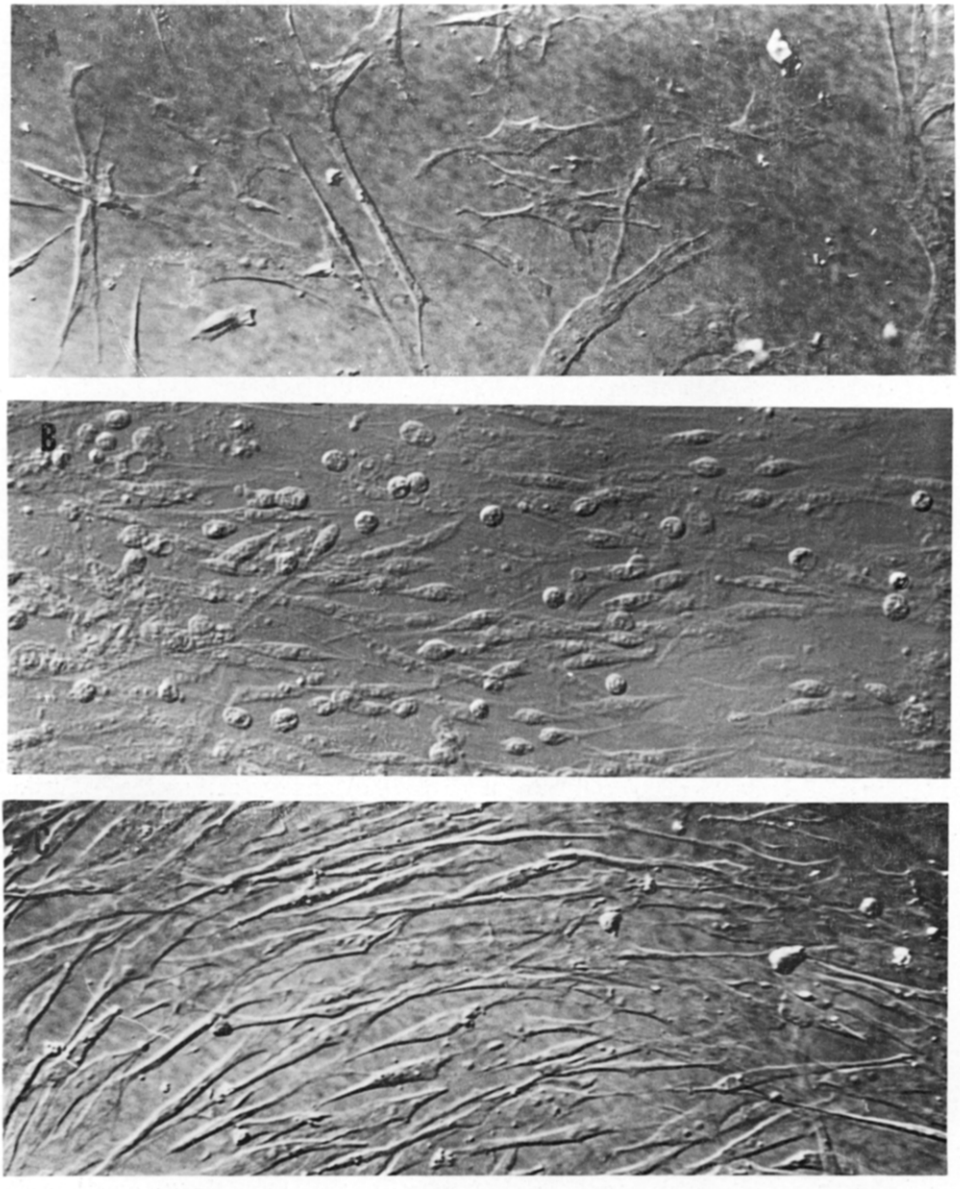

Fig. 1. Nomarski light-interference photomicrographs of cultured chick embryo fibroblasts. A. Uninfected cells. B. Chick embryo fibroblasts which have been infected with the avian sarcoma virus, RBA. Conversion of fibroblasts by this oncogenic virus leads to morphological shape changes (*e.g.*, fusiform-shaped cells to round ones). However, fusiform-shaped cells are always present in these cultures. C. Fibroblasts which have been infected with the leukosis virus, RAV-49. These cells are fusiform in shape. × 28.

RESULTS

Light and electron microscopic studies

Infection and subsequent conversion of chick embryo fibroblasts by RBA virus produces a change in the morphology of the cells. The control fibroblasts are fusiform in shape (Fig. 1A), while the sarcoma virus-converted cells assume a round shape

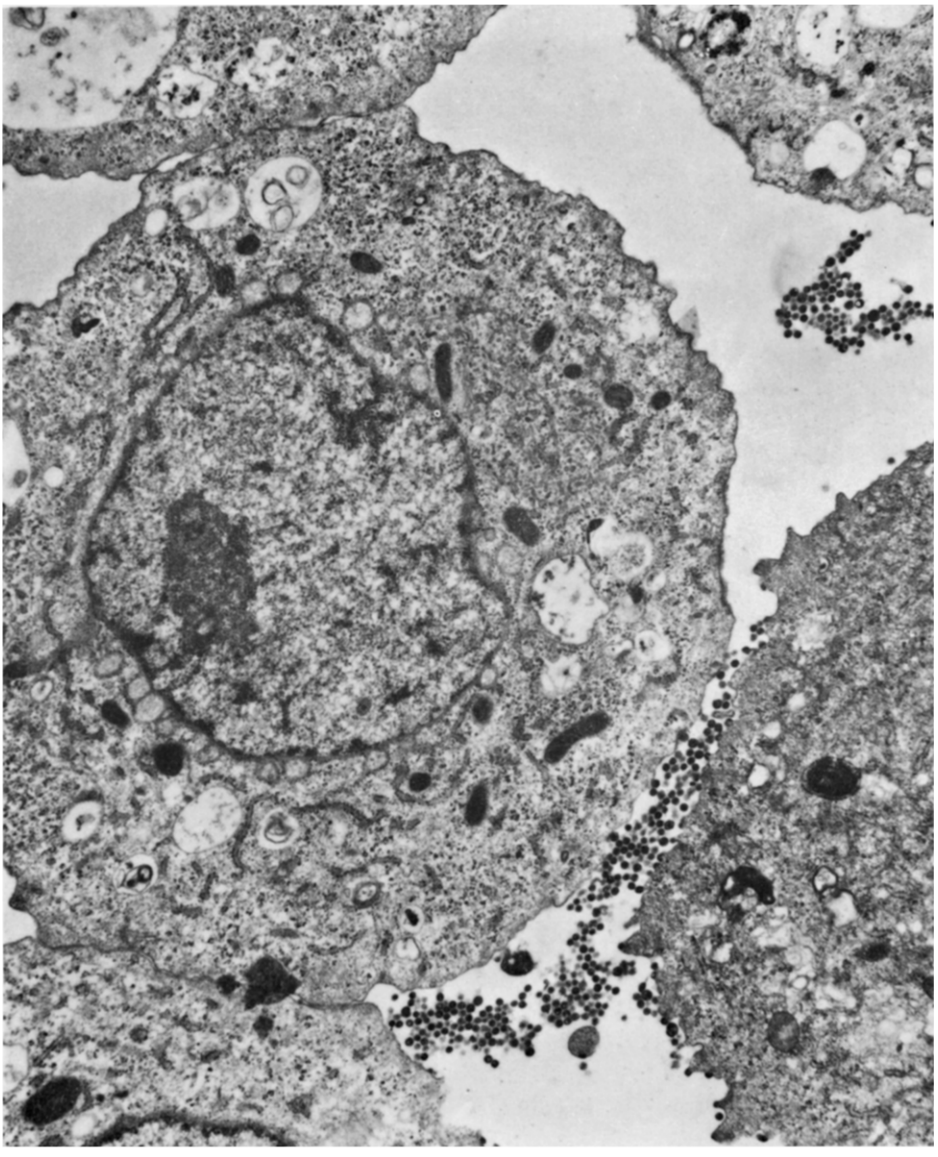

Fig. 2. A photoelectron micrograph of a thin section from chick embryo fibroblasts which have been converted by RBA. The round shaped cells have numerous free polysomes concomitant with a decrease in rough endoplasmic reticulum. Intercellular junctions are established by the converted cells with a gap of between 150 and 400 Å. Virus particles, 800–1000 Å in diameter, are formed by budding from the cell membrane. × 5000.

with cells piling one upon another (Fig. 1B). However, not all the virus-infected cells round up, and there remain within the culture a high percentage of cells which are fusiform in appearance. Cells of either shape produce virus by budding from the surface (Fig. 2).

The intracellular membranes of the round-shaped cells are decreased in number concomitant with an increase in free polysomes. These cells form intercellular junctions (Fig. 2), many of which are longer than has been observed in cultures of uninfected

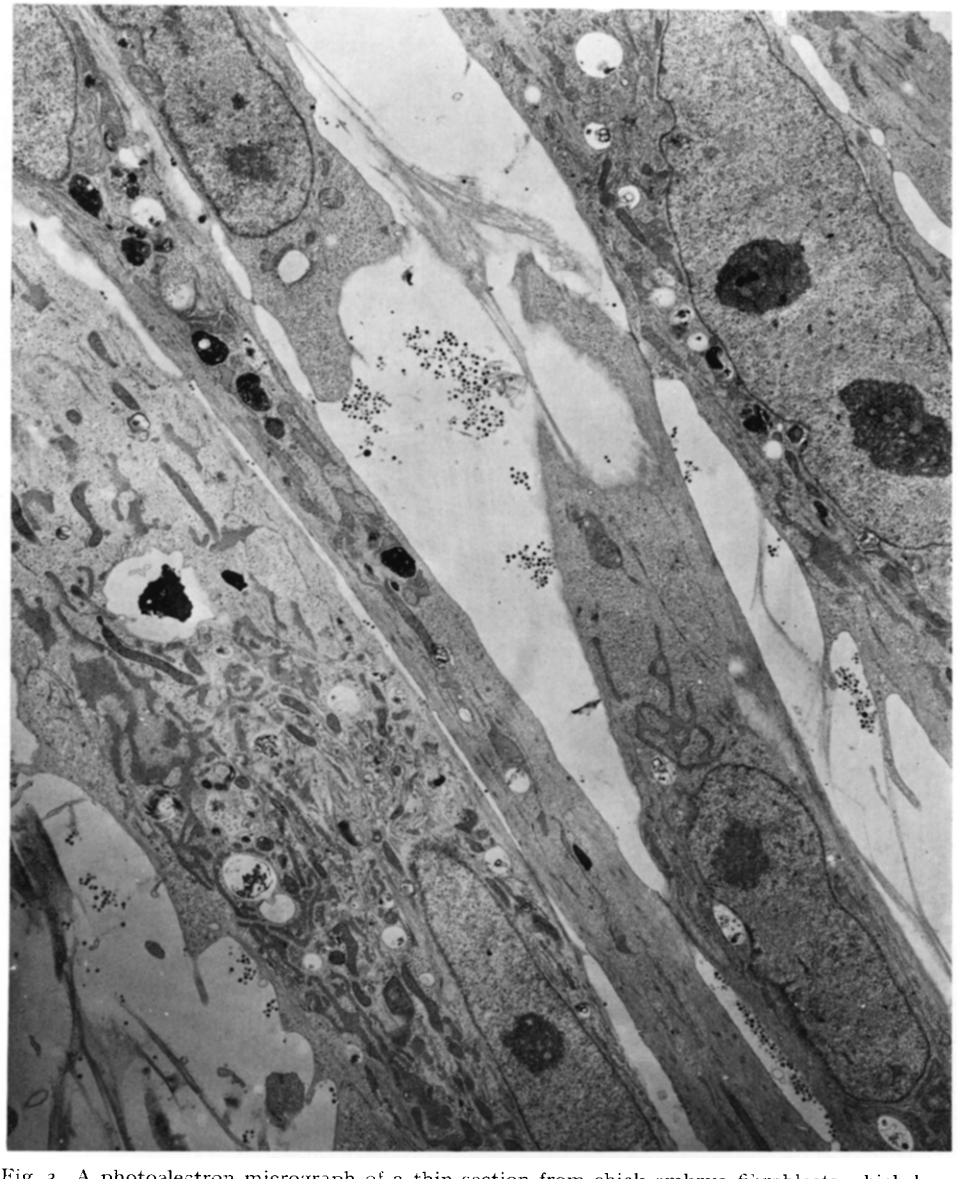

Fig. 3. A photoelectron micrograph of a thin section from chick embryo fibroblasts which have been infected by RAV-49. These cells are fusiform in shape and have a highly developed endoplasmic reticulum. C-type RNA viruses are produced by budding from the cell surface. × 3400.

fibroblasts. The distance between these cells has varied between 140 and 300 Å.

Cultured fibroblasts that have been infected with the RNA-leukosis virus, RAV-49, maintain their fusiform shape (Fig. 1C and Fig. 3). C-type RNA virus is also produced and released by these cells by budding at the surface. Although both kinds of infected cells are producing virus, very definite morphological and possibly cell adhesion differences exist between them, which may be reflected in an altered cell surface chemistry.

The isolation of plasma membrane

The distribution of the material present in the particulate homogenate on continuous density gradients of sucrose gave the banding pattern presented in Fig. 4. The A and B bands were found to be concentrated in cell membrane components[16] and were recentrifuged on the second gradient to give the A′ and B′ fractions. There was no distinctive difference in the banding patterns of these membranes between the converted and uninfected cells. The refractive index measurement for the A′ band was 1.355 ± 0.002 (S.D.) for the control cultures, 1.356 ± 0.002 for RBA-converted cells, and 1.357 (two determinations) for the membrane isolated from RAV-49 infected cultures. The lower fractions on the gradient, designated as C and D in the previous work[16], were combined for this study.

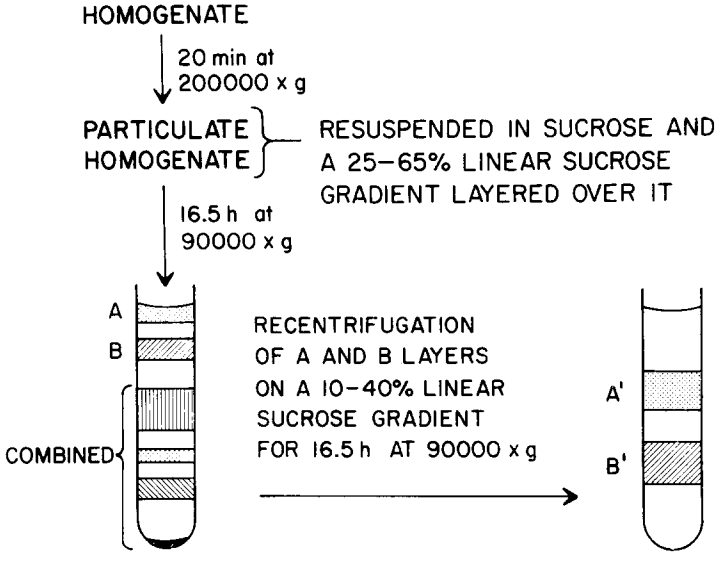

Fig. 4.Procedures used to isolate plasma membrane from chick embryo fibroblasts and fibroblasts infected with RBA and RAV-49.

The biochemistry of fractions isolated from uninfected and virus-infected cultures

The quantification of membrane components, *e.g.* protein, sialic acid, RNA, *etc.*, present in the A′, B′, and combined fractions represented 60–90 % of the material present in the particulate homogenate (Table I). The membrane in the A′ bands from both uninfected and oncogenic virus-converted cells accounted for 2% of the protein and 5–13% of the previously designated[16] plasma membrane components (*e.g.*,

TABLE I

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

Component or enzyme		*Homogenate*
		Units/mg protein
CTPase (μmoles P_i released per 30 min)	Fibroblasts	0.73 ± 0.03**
	RBA-converted cells	0.88 ± 0.13
Cytochrome *c* reductase (μmoles reduced per min)	Fibroblasts	0.024 ± 0.005
	RBA-converted cells	0.011
Succinate dehydrogenase (μmoles indophenol reduced per min)	Fibroblasts	0.007 ± 0.001
	RBA-converted cells	0.009 ± 0.004
Sialic acid (nmoles)	Fibroblasts	21 ± 3
	RBA-converted cells	18 ± 2
Glucose equivalents (μg)	Fibroblasts	127 ± 19
	RBA-converted cells	132 ± 17
Phospholipid (μg)	Fibroblasts	320 ± 15
	RBA-converted cells	291 ± 21
Cholesterol (μg)	Fibroblasts	105 ± 8
	RBA-converted cells	95 ± 7
RNA (μg)	Fibroblasts	75 ± 10
	RBA-converted cells	88 ± 11
DNA (μg)	Fibroblasts	22 ± 2
Protein (mg)	Fibroblasts	
	RBA-converted cells	

* The percent distribution of the membrane components present in the homogenate (100%).

** The calculated means ± S.E. are based upon the combined results of 4 to 11 experiments with two preparations.

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

CTPase, sialic acid, phospholipid, and cholesterol), relative to the particulate homogenate as 100%. The specific activity of CTPase was lower in these series of experiments than had been observed previously[16]. The specific concentrations of the other cell membrane components remained about the same. One exception to this generalized reproducibility was the quantity of RNA that was present in the isolated A′ band. The 36 μg per mg protein was much higher than had been obtained in earlier preparations[16] and represented about 1% of the initial RNA.

The degree of purity of the RBA-converted cell membrane preparation was as good as was achieved for the uninfected cultures. The specific concentrations of phospholipid and cholesterol were the same for the two preparations, but significant differences were found in the specific concentrations of sialic acid and neutral sugars.

The sialic acid content of the membranes in the A′ band from the oncogenic virus-converted cells was decreased in its specific concentration from 106 nmoles per mg protein to 79 nmoles per mg membrane protein (Table I). The quantity of sialic

B′, AND THE COMBINED C AND D, FOLLOWING CENTRIFUGATION OF HOMOGENATES OF CHICK FIBROBLASTS

Band A′		*Band B′*		*Combined C and D bands*		% *Recovery*
Units/mg protein	%*	*Units/mg protein*	%	*Units/mg protein*	%	
1.78 ± 0.28	5 ± 2	2.32 ± 0.24	26 ± 3	0.37 ± 0.03	40 ± 5	71
2.67 ± 0.19	5 ± 2	1.45 ± 0.29	19 ± 2	0.24 ± 0.03	34 ± 7	58
0.00	0	0.017 ± 0.011	2 ± 1	0.026 ± 0.009	64 ± 7	66
0.00	0	0.004	1	0.007	63	64
0.00	0	0.003 ± 0.003	2 ± 2	0.006 ± 0.001	59 ± 7	61
0.00	0	0.00	0	0.006 ± 0.003	60 ± 11	60
106 ± 3	7 ± 2	79 ± 3	21 ± 3	9 ± 1	48 ± 4	76
79 ± 3	5 ± 1	82 ± 9	21 ± 3	9 ± 1	38 ± 4	64
243 ± 8	3 ± 1	153 ± 6	8 ± 2	121 ± 6	61 ± 7	72
358 ± 12	4 ± 1	178 ± 10	8 ± 2	106 ± 14	65 ± 9	77
1998 ± 63	9 ± 1	916 ± 43	19 ± 2	176 ± 14	44 ± 3	72
1979 ± 98	11 ± 2	853 ± 61	18 ± 1	152 ± 12	44 ± 5	73
613 ± 46	10 ± 2	403 ± 23	28 ± 3	46 ± 4	35 ± 3	73
619 ± 12	13 ± 3	351 ± 23	20 ± 2	50 ± 5	35 ± 6	68
36 ± 3	0.7	26 ± 5	2 ± 1	60 ± 11	58 ± 7	61
23 ± 9	0.4	35 ± 10	6 ± 3	54 ± 6	57 ± 5	64
7 ± 3	0.6	6 ± 2	3 ± 2	34 ± 4	89 ± 3	93
0.40§ ± 0.05	2 ± 1	2.26§ ± 0.07	7 ± 1	26.45§ ± 3.47	79 ± 3	88
0.23 ± 0.06	2 ± 1	1.09 ± 0.34	8 ± 1	10.73 ± 2.01	81 ± 8	91

the exception of cytochrome *c* reductase from the RBA-converted cells, which was measured on only

acid in the membranes from the B′ band were the same for both preparations. In contrast to this viral-induced decrease in sialic acid, there was a significant increase in the quantity of neutral sugar associated with membranes from both the A′ and B′ band from the RBA-infected cells. In the A′ band, neutral sugar was increased from 248 to 358 μg per mg membrane protein.

Cell membrane was isolated from chick embryo fibroblasts that had been infected with the leukosis virus, RAV-49. The sialic acid content of the membrane in the A′ band was higher than that of the uninfected controls (Table II), rather than lower as was observed with RBA-infected cells. The neutral sugar content of this membrane was in the range found for the control cells.

The relationship of cell membrane chemistry to cell shape and to conversion by avian RNA oncogenic virus is currently being studied[28]. In this connection, there were four experiments in which chick embryo fibroblasts had been infected with titers of RBA-virus that should have produced morphological conversion but that for un-

TABLE II

THE DISTRIBUTION OF PROTEIN, SIALIC ACID AND NEUTRAL SUGAR AMONG BANDS A′, B′, AND THE COMBINED C AND D, FOLLOWING CENTRIFUGATION OF HOMOGENATES OF RAV-49-INFECTED FIBROBLASTS ON CONTINUOUS DENSITY GRADIENTS OF SUCROSE

Component	*Homogenate*	*Band A′*		*Band B′*		*Combined C and D bands*		% *Recovery*
	Units/mg protein	*Units/mg protein*	%*	*Units/mg protein*	%	*Units/mg protein*	%	
Protein (mg)		0.69** ± 0.14***	2 ± 1	3.08 ± 0.13	6 ± 1	44.23 ± 15.89	78 ± 4	86
Sialic acid (nmoles)	26 ± 3	114 ± 3	6 ± 2	92 ± 3	24 ± 4	12 ± 1	42 ± 4	72
Glucose equivalents (μg)	110 ± 21	198 ± 20	2 ± 1	138 ± 13	7 ± 2	98 ± 14	82 ± 7	91

* The percent distribution of the membrane components present in the homogenate (100%).

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

*** The calculated means ± S.E. are based upon the combined results of 5 experiments.

known reasons did not. Plasma membrane was isolated from these cells, and the sialic acid concentration of the A′ band material was 104 nmoles per mg protein. The neutral sugar content of the A′ band membrane was 263 μg per mg protein. These values for membrane components were like those of uninfected controls.

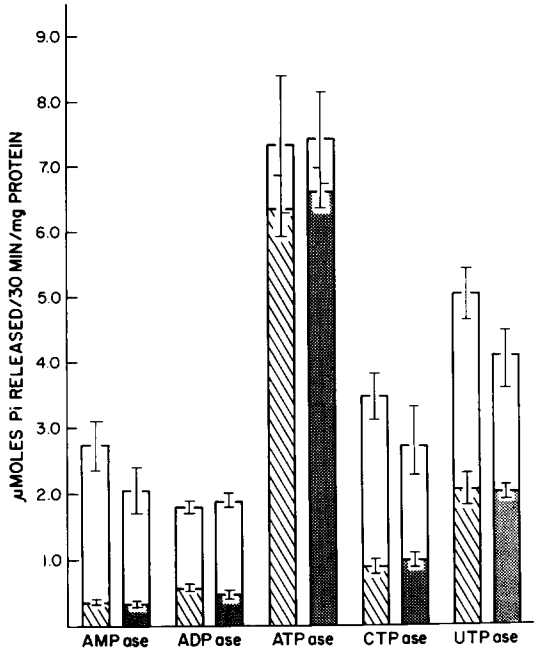

Fig. 5. The phosphohydrolase activities of the particulate homogenate and membranes in the A band from uninfected and RBA-converted fibroblasts. Membrane fractions from the particulate homogenate and A band from uninfected cells (▨, □) and from RBA-converted cells (▩, □), respectively, were incubated at 37° 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 calculated means ± S.E. are based on the results of four experiments.

The phosphohydrolase activity of isolated cell membranes

The specific activities of the nucleotide phosphohydrolase, known to be associated with the plasma membrane[16,22], were determined for plasma membrane isolated from uninfected and from RBA-converted cultures. There was no significant difference between these preparations with any of the nucleotide substrates except for UTPase, which was decreased in the virus-converted cells (Fig. 5). There were also decreases in the specific activities of AMPase and CTPase, but they were not significant. The quantity of ATPase in the membranes in the B′ band was greater than it was in those in the A′ layer. The specific activity of the Mg^{2+}-ATPase was 11.80 ± 1.70 for the B′ but 7.37 ± 1.05 for the A′ band. Both preparations were stimulated by K^+, 8.64 for the A′ and 13.95 for the B′ band. This activity was inhibited by ouabain.

Morphology of isolated plasma membrane

The membrane in the A′ band was examined in negative contrast with the expectation of finding surface subunits, organized like those which have been ob-

Fig. 6. A photoelectron micrograph of membranes in the A′ band from uninfected fibroblasts which have been stained with phosphotungstic acid. The membranes in this fraction had to be fixed with glutaraldehyde and osmium in order to prevent the formation of myelin-like figures. There was no discernible subunit organization of the membrane or differences between uninfected and RBA-converted cell membranes, with this double fixation procedure. × 110000.

served by BENEDETTI AND EMMELOT[29] on the plasma membrane from liver. The membranes from both normal and oncogenic virus-converted cells were examined following fixation with glutaraldehyde and osmium. These membranes have a smooth surface structure with no discernible subunit organization, and no differences were seen between uninfected and virus-infected preparations (Fig. 6). A double fixation of the membranes was required, since glutaraldehyde fixation followed by phosphotungstic acid staining resulted in an artifactitious generation of myelin-like figures.

DISCUSSION

Flotation equilibrium centrifugation of homogenates from uninfected and virus-infected cells (RBA and RAV-49) resulted in similar distributions within the sucrose gradients of plasma membrane and intracellular membranes and organelles. The plasma membrane components were concentrated in the A′ and B′ layers. The specific concentration of lipid was the major difference in these two preparations, with the A′ layer having twice as much phospholipid as the B′ layer. The sialic acid and neutral sugar contents of the membranes in the B′ fraction were lower than those of membranes in the A′ fraction, but the specific activities of the nucleotide phosphohydrolases (Table I) were equal to or greater than those found in the lighter membrane fraction. Morphological differences were previously observed for the membranes in the A band and for those in the B band[16]. The membranes in the B band are vesicular and are smaller in diameter than were membranes from the A band. The latter plasma

membranes were obtained as large sheets from Zn^{2+}-treated cells or as vesicles and large membrane fragments from cells which were homogenized in saline.

There may exist within the cell surface domains having large lipid, protein, and carbohydrate compositional differences. Recent topographical analysis of the H-2 antigens of mouse thymocytes and lymphocytes disclosed that these cell membrane components were restricted to discrete regions of the cell surface[30]. It is possible, therefore, that during the homogenization of the cells, membrane fragments of unique composition, such as those found in the A′ and B′ bands, could be formed by separating the cell membrane at the boundaries of these chemically distinctive regions. Recently, it has been reported by EVANS[31] and BARBER AND JAMIESON[32] that the plasma membrane from liver and human blood platelets can be separated into two distinctive fractions.

The membranes in the A′ fraction that were isolated from the RBA-converted cells had a buoyant density, lipid composition, and morphology like those of cell membranes from the uninfected cells. However, the virus-converted cells were different from the uninfected controls in the levels of neutral sugar and sialic acid which were associated with the isolated cell membranes. Neutral sugar was increased in the RBA-converted cell membranes but less sialic acid was present in these membranes. The membranes in the B′ fraction from the virus-converted cells were also higher in neutral sugar, while sialic acid concentrations were unchanged.

Changes in the carbohydrate content of the cell surface in neoplasms or in cells transformed by oncogenic virus have been reported by many investigators[33]. DEFENDI AND GASIC (quoted in ref. 33), using colloidal iron, noted an increase in the staining of the cell surface of polyoma virus-transformed cells. The staining reaction was sensitive to treatment with neuraminidase. Staining virus-transformed cells with ruthenium red gave similar results[33]. In Rous Sarcoma Virus-transformed cells, MORGAN[34] found no correlation between thickness of the ruthenium red layer and the degree of contact inhibition. The stained material was insensitive to neuraminidase but could be removed by treatment with hyaluronidase. It had been reported by TEMIN[35] and ISHIMOTO, *et al.*[36] that Rous Sarcoma-Virus-converted cells synthesized higher levels of hyaluronic acid, but TEMIN[35] also found that leukosis and myoblastosis virus infections of fibroblasts did not lead to greater levels of hyaluronic acid.

The contribution of sialic acid for surface charge, enzymatic activities, intercellular adhesion and recognition, *etc.*, has been established by investigations over the past 10 years (this subject has recently been reviewed by WINZLER[37]). Tumors, arisen by spontaneous transformation or produced by oncogenic virus, were frequently found to have an increased net negative charge as evidenced by mobility measurements. In addition, there was some evidence for an increase in sialic acid in membranes isolated from cancer cells. LI *et al.*[38] found levels of sialic acid and hexosamines in the mitochondrial, microsomal, and lysosomal fractions of Walker 256 tumors which were higher than those of rat livers. EMMELOT AND BENEDETTI[39] also observed an increase in sialic acids and neutral sugar in a hepatoma from the rat, 484, but no difference in the mouse Hepatoma 143066.

More recent studies, using virus-transformed cells, indicate that the cellular levels of sialic acid are decreased. OHTA *et al.*[40] observed that polyoma virus-transformed cells had less sialic acid than did the uninfected cells. Other laboratories, in particular those of HAKOMORI[41-44], BRADY[45,46], WARREN[47], and ROBBINS[48,49], have

shown that viral transformation results in sialic acid decreases and changes in the carbohydrate composition of glycolipids and glycoprotein fractions. HAKOMORI has proposed that virus transformation results in the synthesis of incomplete glycolipids, *i.e.*, the terminal and branched sialic acids are not added to the other carbohydrate. New properties of the membrane, including intercellular aggregations and the emergence of antigens, become evident. The absence of specific glycosyl transferases may account for these incomplete carbohydrates. GRIMES[50] found a 55–60 % decrease in the quantity of sialyl transferase in SV-40-transformed 3T3 cells, which had a 60 % decrease in sialic acid.

Sialic acid decreases may reflect the inability of rapidly growing normal or cancer cells to complete carbohydrate synthesis. GLICK, *et al.*[51] observed that KB cells synthesized carbohydrates, including sialic acid, in synchrony with the cell cycle. These investigators[52] found that the degree of confluency influences the quantity of sialic acid associated with the cells. HAKOMORI[43] observed that glycolipids were incomplete in normal cells that were dividing rapidly.

The cells used in this study were always in the stationary phase when harvested for membrane isolation. We have noted one inconsistency in the sialic acid content of the cell membrane. Four preparations of cell membrane obtained from embryos in the spring of 1970 had values for sialic acid which were much lower than those reported in Table I. The uninfected cell membrane contained 66 $\pm$ 4 (S.E.) nmoles of sialic acid per mg protein. Cell membrane obtained from RBA-converted cells had 38 $\pm$ 3 nmoles of sialic acid. The other cell membrane components were unchanged.

The increase in carbohydrate in the cell membrane of virus-converted cells has been observed previously. TEMIN reported that avian sarcoma-converted cells had levels of hyaluronic acid in medium which were much higher than those of controls, and the cell membranes from Hepatoma 484 and the Walker 256 tumor had increased neutral and amino sugars compared with those of rat liver. WU *et al.*[48] found a decrease in neutral and amino sugar in SV-40-transformed 3T3 cells, but MEEZAN *et al.*[49], using the same cell system, found a higher molecular weight glycopeptide in fractions released with pronase and separated by Sephadex chromatography. BUCK *et al.*[47], using BHK 21/C13 cells, found that fucose was incorporated into a higher molecular weight glycopeptide fraction following transformation of these cells with Rous Sarcoma Virus. More recent work[53, 54] has extended the observations to polyoma and murine sarcoma virus-transformed cells.

Plasma membrane that was isolated from the leukosis virus-infected cells (RAV-49) had values for sialic acid and neutral sugar similar to those of the control cell cultures. Yet, these cells were infected with an RNA virus of the same subclass as the RBA and produced virus particles by budding from the cell surface. It may be inferred that the changes in cell membrane chemistry observed in the RBA-infected chick embryo fibroblasts were not likely to be the result of virus infection or virus maturation at the cell surface. Rather, these biochemical changes are phenotypic expressions of conversion by an oncogenic virus and are virus-directed alterations in cell membrane chemistry. This increase in the neutral sugar level of the plasma membrane, although controlled by oncogenic viruses, is not necessary for the expression of the oncogenic state. Studies[28] on the chemistry of the avian sarcoma viruses, morph^r and morph^f Fujinami, indicate that the converted cells which are fusiform in shape do not have elevated levels of neutral sugar. However, both cell types, the round

and fusiform, have cell membranes with decreased amounts of sialic acid. The latter membrane change is produced by all avian oncogenic viruses which have been examined. The modifications of the sites or dispositions of this carbohydrate may result in cell surfaces which are less adhesive, have new antigenic properties, and are deficient as transducers of environmental signals that regulate cell growth.

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