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PROTEINS AND GLYCOPROTEINS IN PLASMA MEMBRANE FRACTIONS OF AVIAN LEUKOSIS–SARCOMA VIRUS SUSCEPTIBLE AND RESISTANT CHICKEN EMBRYO FIBROBLASTS

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SUMMARY

^{3}H- or ^{14}C-labeled proteins and glycoproteins from plasma membrane-enriched fractions of avian leukosis virus susceptible and resistant chick embryo fibroblasts were solubilized with sodium dodecyl sulfate and compared after polyacrylamide gel electrophoresis at pH 9.0. No apparent differences in electrophoretic patterns were observed when both phenotypes were cultured in the presence of radioactive leucine or glucosamine. A major leucine-rich polypeptide of apparent mol. wt 42000 was common to both cell types whereas most of the glycoproteins components were found in the mol. wt range 82000–110000. Leucine, glucosamine and fucose were present in a major low molecular weight component which migrated near bromophenol blue. Limited studies comparing fucose-labeled proteins suggested that phenotypic differences may occur in plasma membrane fucoproteins.

INTRODUCTION

Resistance to infection of chicken cells by avian leukosis–sarcoma (L–S) viruses belonging to subgroups A and B is controlled, in each case, by a single, autosomal-recessive gene[1]. The block to infection apparently occurs at an early stage, since resistant cells neither produce infective virus nor become transformed. Crittenden[2] further showed that resistance occurs at the level of virus penetration or uncoating because normally resistant cells can, under special experimental conditions, produce progeny virus of the excluded subgroup if the viral genome enters the cell. Since susceptibility is dominant, it was suggested that the presence of a specific cell surface associated factor may be necessary for virus infection. This report describes attempts to extend the biological evidence for susceptiblity using biochemical techniques.

Although the electrophoretic distribution of solubilized membrane proteins has been studied extensively from a variety of mammalian cells, little has been reported concerning polyacrylamide gel electrophoretic patterns of proteins derived from chick embryo fibroblast membranes. We compared proteins and glycoproteins

Abbreviation: L–S virus, avian leukosis–sarcoma virus.

obtained from enriched membrane fractions of virus susceptible and resistant fibroblasts originating from matings of highly inbred chickens (Line 100). Since this line is segregating for genes controlling resistance to infection by subgroups A and B of the L–S viruses[1], it provides a unique model for the *in vitro* exploration of genetically controlled plasma membrane–virus interactions free from immunologic or hormonal influences.

EXPERIMENTAL PROCEDURES

Cultivation and labeling of cells

Embryos susceptible to infection by L–S viruses of subgroups A and B (C/O) and resistant to virus subgroups A and B (C/AB) were obtained from selected matings. Susceptibility phenotypes were determined by challenge of primary cultures with Rous sarcoma virus of subgroups A and B[3]. Both phenotypes were negative for endogenous virus production when tissue culture fluids from uninfected cells were examined using the RNA-dependent DNA polymerase assay[1]. Cells from the same two embryos were compared throughout this study. Stock suspensions were stored in liquid nitrogen and cultured in maintenance medium, containing equal parts of 199 and F-10 media and supplemented with 5% tryptose phosphate broth, 2% fetal calf serum, 2% calf serum, and 5% bovine amniotic fluid[5].

In double-labeling experiments using leucine, two sets of 20 Falcon tissue culture dishes (100 mm diameter) each containing non-radioactive medium, were seeded with $4 \cdot 10^6$ cells per plate of either the virus susceptible or resistant phenotype. After 24 h, 10 dishes containing either phenotype were divided into a subset and 8 ml of Eagle's minimal essential medium were added containing one-tenth the normal amount of amino acids and supplemented with L-[U-^{14}C]leucine (262 mCi/mmole) at a final concentration of 0.34 μCi/ml. To the remaining subset, 8 ml of media containing 1.7 μCi/ml of L-[4,5-$^3H_2$]leucine (38.6 Ci/mmole) were added, and both subsets were incubated for an additional 48 h at 38 °C. Isotopic labeling of cells was reversed in the remaining set and cultures were incubated concurrently.

When labeled glucosamine was used, cultures were prepared as described above, but were subsequently grown for 48 h in 8 ml of maintenance medium containing D-[1-^{14}C]glucosamine (57 mCi/mmole) at 0.59 μCi/ml or D-[6-^{3}H]glucosamine (3.6 Ci/mmole) 1.7 μCi/ml.

Labeling of cells with L-[^{3}H]fucose (4.8 Ci/mmole) or L-[1-^{14}C]fucose (56.2 mCi/mmole) was accomplished during 48 h incubation in maintenance medium containing 3.0 μCi/ml [^{3}H]fucose or 0.25 μCi/ml [^{14}C]fucose. Radioactive precursors were purchased from New England Nuclear*.

Membrane preparation

Labeled cell layers were washed twice with isotonic saline before removal by scraping with a rubber policeman, and suspensions were centrifuged at $4000 \times g$ for 10 min. Labeled suspensions of virus susceptible and resistant cells were pooled, allowed to swell in 8–10 ml of a hypotonic solution consisting of 0.01 M Tris–HCl (pH 7.2) containing 0.001 M $MgCl_2$, and disrupted with 15–30 strokes

* Use of a company or product named by the Department does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

of a Type B pestle in a Dounce homogenizer. Cell rupture was monitored using phase-contrast microscopy. After centrifugation at $4000 \times g$ for 10 min to remove whole cells and nuclei, supernatants were further centrifuged at $17000 \times g$ for 30 min. The pellets, containing plasma membranes and internal organelles, were resuspended in 3–5 ml Tris–HCl–$MgCl_2$ buffer and placed on top of a discontinuous gradient composed of four layers, containing 8 ml each of 50, 40, 30, and 20% (w/w) sucrose. Tubes were centrifuged for 18 h at 23000 rev./min in a Spinco SW-27 rotor. Cell rupture and subsequent procedures were conducted at 2–5 °C.

Turbid bands of densities (*d*) 1.05, 1.10, 1.14, 1.18, and 1.22 g/ml were collected with pasteur pipettes and refractive indices were measured in an Abbe refractometer. Bands were diluted with 8–10 vol. of water, and centrifuged at $29000 \times g$ for 30 min. Pellets from light fractions were washed once with cold 0.5 M NaCl to release trapped contaminants, and after centrifugation, fractions were finally resuspended in 0.5 ml cold water. Samples not taken for electron microscopic observation, protein content[6], CTPase activity[7], or acrylamide gel electrophoresis were stored at either −20 °C or −70 °C.

Electron microscopy

Membranes were fixed overnight with 3% glutaraldehyde, rinsed three times with phosphate buffer, and fixed with 1% OsO_4 for 1 h. Preparations were dehydrated by sequential washing with absolute ethanol and propylene oxide, and finally imbedded in Epon-Araldite. Thin sections were cut with a Porter-Blum MT2 ultramicrotome, stained with uranylacetate, and examined in a Phillips EM-200 electron microscope.

Sodium dodecyl sulfate disc gel electrophoresis of plasma membranes

The procedure for solubilization of membranes was described by Glossman and Neville[8]: suspensions (0.1–0.2 ml) were dissolved by the addition of an equal volume of 0.1 M Na_2CO_3 containing 2% sodium dodecyl sulfate. After reduction with 10% β-mercaptoethanol, solubilized membranes were dialyzed anaerobically in a desiccator overnight at 4 °C against upper gel buffer containing dithiothreitol (0.05%), sodium dodecyl sulfate (0.1%), bromophenol blue (0.1%), and sucrose (2%). Dialyzed preparations were heated at 100 °C for 20–60 s, and 0.1–0.2 ml were layered on top of gel columns (0.5 cm diameter) consisting of 1.9 ml separating gel and 0.5 ml stacking gel. The borate–sulfate discontinuous buffer system calculated from theory by Jovin *et al.*[9] was used as modified by Neville[10] with the addition of 0.1% sodium dodecyl sulfate. Samples underwent electrophoresis at 25 °C in 11% gels (8 cm long) with an acrylamide:bisacrylamide ratio of 110. 1 mA per gel was applied for 2.5 h or until the tracking dye was approximately 1 cm from the bottom of the column.

After extrusion from tubes with a threaded plunger, gels were sequentially sliced with a razor blade into sections (approx. 1 mm), and dissolved overnight in 0.5 ml 30% H_2O_2 containing 1% NH_4OH at 37 °C.

Radioactivities were measured in vials containing scintillation counting fluid using a Packard Spectrometer (Model-3375). An IBM-360 (Model 50) computer was used at the Automatic Data Processing Center of the National Agricultural Library for the analysis of radioactivities in gel slices from double-labeling experi-

ments[11]. Figures represent computer drawn plots where cpm in each slice are represented on the ordinate as a percent of the total counts obtained. Slice 1 represents percent counts in the upper gel. The program corrected for the ^{14}C overlap into the

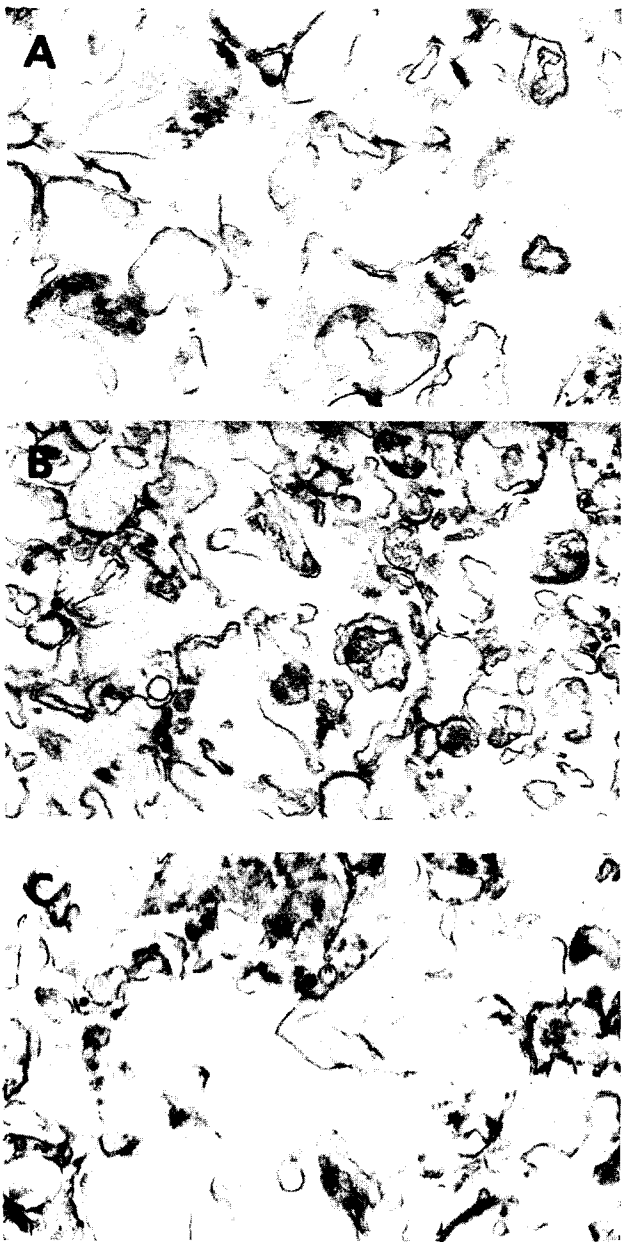

Fig. 1. Photoelectronmicrographs of chick fibroblast plasma membrane enriched fractions after centrifugation for 18 h at 23000 rev./min in a discontinuous sucrose density gradient (20-50%, w/w). Thin sections of material in pellets obtained from three turbid bands of densities; (A) 1.05 *d*, (B) 1.10 *d* and (C) 1.14 *d* are shown. × 32500.

^{3}H channel, whereas the extent of ^{3}H cross-over into the ^{14}C channel was insignificant. Counting efficiency for ^{3}H and ^{14}C was 19% and 42%, respectively.

Reference proteins were labeled by methylation with [^{14}C]dimethylsulfate (New England Nuclear, 1.5 mCi/mM) as described by Kiehn and Holland[12]. β-Galactosidase, DNAase I and avidin were obtained from Worthington Biochemicals: bovine serum albumin was a product of the Sigma Chemical Company, and ovalbumin was purchased from Nutritional Biochemicals. Apparent molecular weights were calculated by interpolation from a smooth curve which was obtained by plotting $-100 \log M$ *vs* molecular weight of marker proteins[10].

RESULTS

Electronmicrographs (Fig. 1) of plasma membrane fractions prepared without treatment with stabilizing agents were shown to consist predominately of large membrane sheets and microvesicles. As expected, electron micrographs of material banding at sucrose densities of 1.18 and 1.22 *d* indicated the presence of rough endoplasmic reticulum, mitochondria and unidentifiable fragments: thus only proteins in the lighter fractions were solubilized and analyzed.

Data in Table I show that the specific activity of CTPase and fucose incorporation was highest in preparations banding at a sucrose density of 1.10 g/ml. After isopycnic centrifugation, enrichment of these activities compared to material put on top of the sucrose gradient was 3.8-fold and 3.4-fold, respectively. With other preparations, the specific activity of ADPase and cholesterol (480 μg/mg protein) content was also observed to be greatest in material banding at this density.

TABLE I

DISTRIBUTION OF CTPase, PROTEIN AND FUCOSE IN FRACTIONS FROM NORMAL, VIRUS SUSCEPTIBLE FIBROBLASTS AFTER DIFFERENTIAL CENTRIFUGATION OF A DOUNCE HOMOGENATE AT 71 300 × *g* AND AFTER ISOPYCNIC CENTRIFUGATION OF PARTICULATE MATERIAL IN A DISCONTINUOUS GRADIENT OF SUCROSE (20–50%, w/w)

CTPase activity is expressed as μmoles P_i, released per mg protein after 30 min incubation. Protein is expressed as total mg obtained from approx. $2 \cdot 10^8$ cells. Fucose is expressed as cpm × 10^{-3} per mg protein.

Component	*Crude fractions*		*Band densities (g/ml)*		
	Supernatant	*Pellet*	*1.05*	*1.10*	*1.14*
CTPase	0.50	0.93	0.71	3.52	1.75
Protein	19.0	26.4	0.17	0.42	1.12
Fucose	34	162	214	550	270

In dual-labeling experiments using leucine, when cells were cultured in the presence of one-tenth the normal amount of amino acids, only one turbid band in the density region of 1.14 g/ml was obtained. The pattern obtained after coelectrophoresis of non-delipidated membranes is shown in Fig. 2, and the apparent molecular weights of protein subunits are listed in Table II. There appeared to be

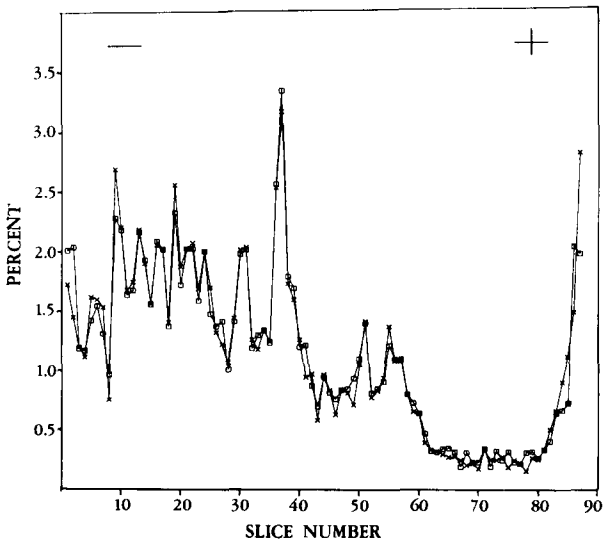

Fig. 2. Sodium dodecyl sulfate acrylamide gel coelectrophoretic patterns of a non-delipidated membrane fraction (45 μg) obtained from material banding at a sucrose density of 1.14 *d*. Virus susceptible and resistant chick embryo fibroblast were grown in the presence of [^{3}H]leucine (□—□) or [^{14}C]leucine (×—×), respectively. A dialyzed sample in a volume of 0.1 ml underwent electrophoresis on a 11% acrylamide gel column (8.0 cm × 0.5 cm) for 2 h at 1 mA. Corrected, total radioactivities analyzed from ^{3}H- and ^{14}C-containing components were 9248 cpm and 6335 cpm, respectively.

TABLE II

APPARENT MOLECULAR WEIGHTS OF LEUCINE-LABELED PLASMA MEMBRANE PROTEINS OBTAINED BY SODIUM DODECYL SULFATE DISC GEL ELECTROPHORESIS

The material banding at a sucrose density of 1.14 g/ml was solubilized in alkaline 1%, sodium dodecyl sulfate, and reduced with β-mercaptoethanol. Molecular weights were calculated from the pattern shown in Fig. 2 by interpolation using mobilities of purified proteins of known molecular weight. Fraction 11 (Slice 37) represents the major leucine labeled component.

Fraction No.	*Mol. wt*	*Fraction No.*	*Mol. wt*
1	> 130000	9	60000
2	> 130000	10	53000
3	125000	11	42000
4	102000	12	33000
5	90000	13	24000
6	80000	14	21000
7	72000	15	< 10000
8	66000		

no striking qualitative or quantitative difference in polypeptide patterns between phenotypes: chick embryo fibroblast membranes contain major leucine-rich subunits of apparent molecular weights of 42000 and less than 12000. A similar pattern, not shown, was obtained in a concurrent experiment when virus susceptible and resis-

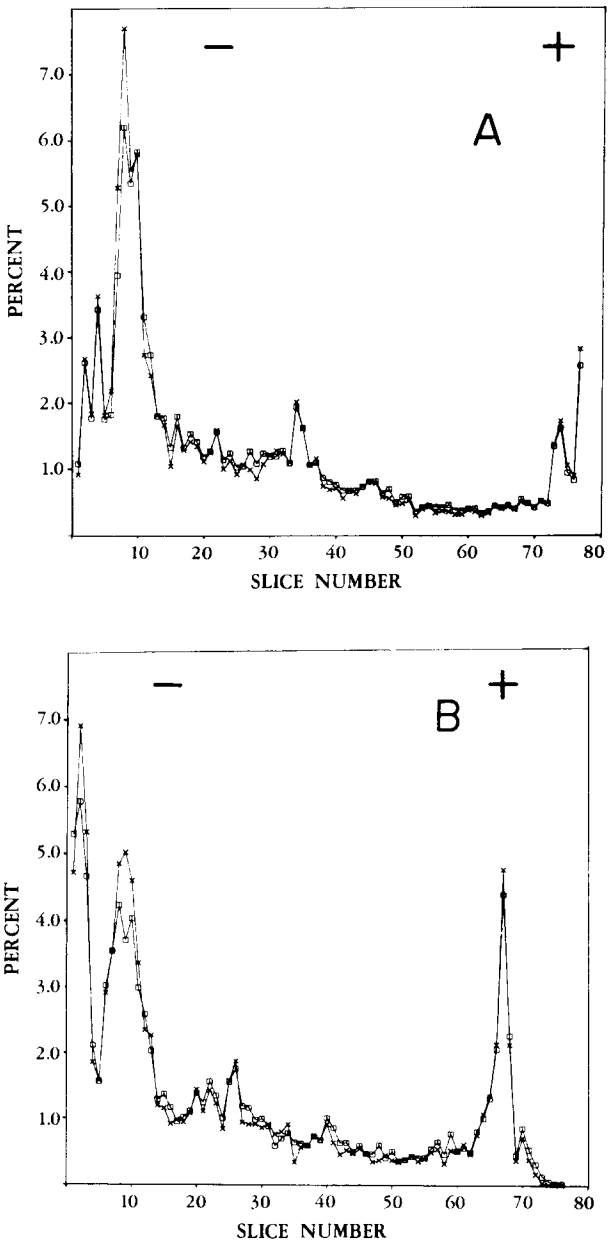

Fig. 3. Patterns obtained from sodium dodecyl sulfate solubilized membrane fractions after susceptible and resistant cells were grown in the presence of [^{3}H]glucosamine (□—□) or [^{14}C]-glucosamine (×—×), respectively. (A) Approximately 45 μg of protein from membranes with a buoyant density of 1.10 *d* underwent coelectrophoresis. Total radioactivities recovered from ^{3}H- and ^{14}C-labeled components were 62150 cpm and 29198 cpm, respectively. (B) Approximately 48 μg of protein from membranes banding at 1.14 *d* underwent electrophoresis. Total radioactivities recovered from ^{3}H- and ^{14}C-labeled components were 18105 cpm and 9790 cpm, respectively.

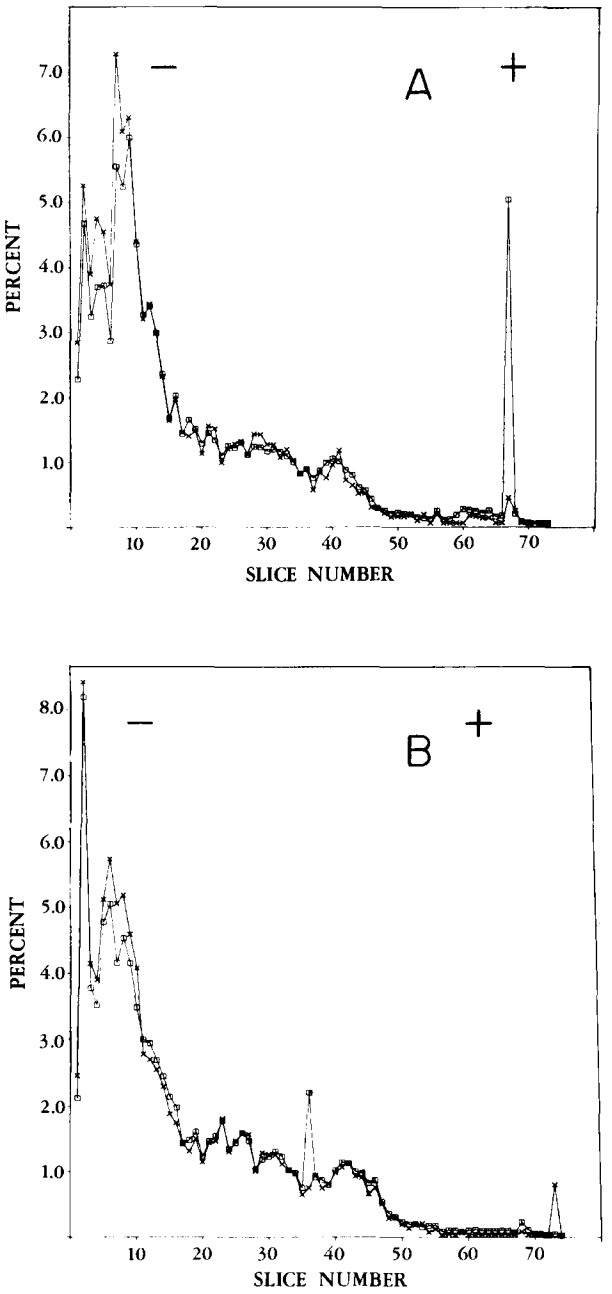

Fig. 4. Coelectrophoresis of freshly prepared, solubilized membrane fractions after virus susceptible and resistant cells were grown in the presence of [^{3}H]fucose (□—□) or [^{14}C]fucose (×—×), respectively. (A) Approximately 216 μg of protein in the 1.10 *d* fraction underwent electrophoresis. Total corrected ^{3}H- and ^{14}C counts analyzed were 12346 cpm and 1514 cpm, respectively. (B) Approximately 240 μg of protein in the 1.14 *d* fraction underwent electrophoresis. Total corrected ^{3}H and ^{14}C counts were 19762 cpm and 2673 cpm, respectively.

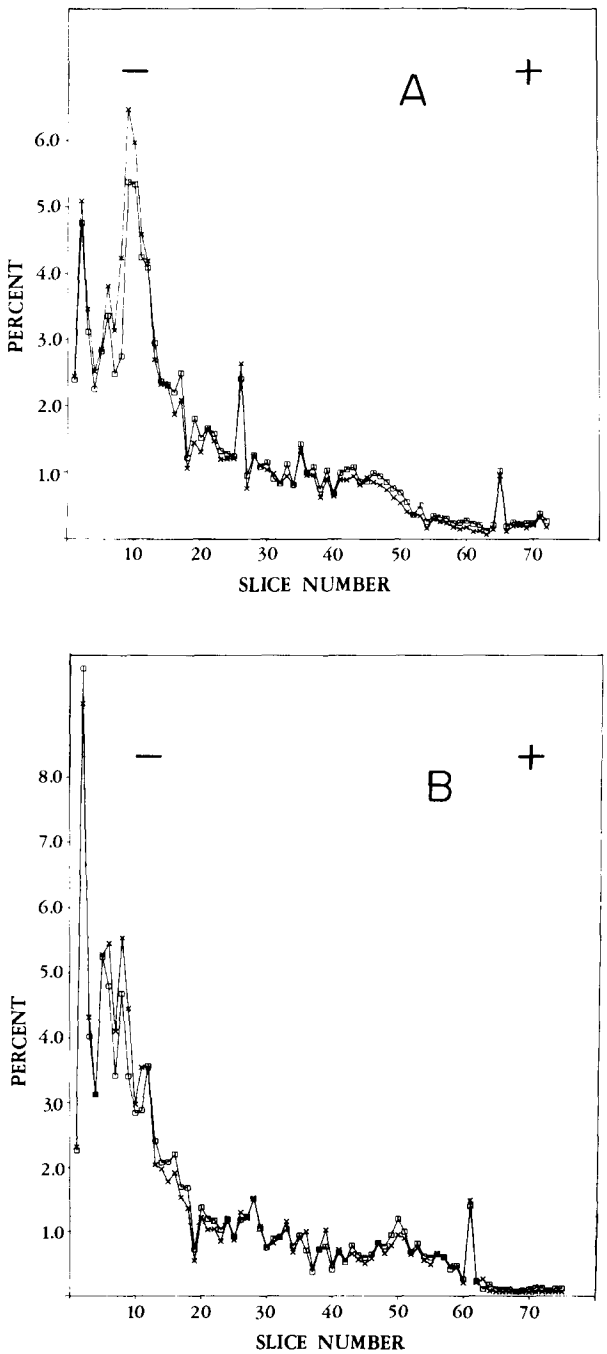

Fig. 5. Coelectrophoresis of fucose-labeled membrane-enriched fractions after storage at −70 °C for one week. (A) Total corrected ^{3}H and ^{14}C counts analyzed in the 1.10 *d* fraction were 23314 cpm and 3467 cpm, respectively. (B) Total corrected ^{3}H and ^{14}C counts analyzed in the 1.14 *d* fraction were 11561 cpm and 1708 cpm, respectively. Preparation notations described in Fig. 4.

tant cells were cultured in the presence of [^{14}C]leucine and [^{3}H]leucine, respectively.

After isopycnic centrifugation of glucosamine-labeled cell homogenates, major plasma membrane enriched fractions appeared in sucrose density regions of 1.10 and 1.14 *d*; again no significant difference in glycoprotein composition was found between phenotypes in either band (Figs 3A and 3B). In contrast to the leucine-labeled pattern, major glycoprotein components of apparent molecular weight 110000 and greater were seen. In addition, fractions banding in sucrose density regions of 1.10 *d* and 1.14 *d* also contained subunits of molecular weight 42000 and 53000, respectively. A similar pattern, not shown, was also found when susceptible and resistant cells were grown in media containing [^{14}C]glucosamine and [^{3}H]-glucosamine, respectively.

Although preparations from both phenotypes consistently showed similar coelectrophoretic patterns with leucine- and glucosamine-labeled proteins, patterns obtained with fucose-labeled components were not reproducible. Using freshly prepared fractions (Fig. 4), we observed a prominent fucose-rich peak near the tracking dye with both phenotypes, and 1–2 distinct single slice peaks were variously found associated with either susceptible or resistant phenotypes. However, when particulate preparations were thawed after storage at −70 °C for one week and subsequently solubilized and dialyzed preparatory to electrophoresis, the presence of low molecular weight components and constituent differences between phenotypes were not apparent (Fig. 5). This phenomenon was observed in earlier experiments, but only with fucose-labeled preparations.

Aside from the lability of fucose-labeled components, overall patterns obtained resembled those found with glucosamine-labeled fractions; most of the radioactivity was associated with proteins of molecular weight interpolated to be in the range of 82000–110000. It should be noted that although there was electron microscopic evidence for the presence of membranes in the 1.05 *d* fraction (Fig. 1) insufficient material was recovered for electrophoretic comparisons in subsequent double-labeled fucose experiments after the small amount of sedimented material was washed with 0.5 M NaCl.

DISCUSSION

Under normal growth conditions, two major chick embryo fibroblast plasma membrane-enriched fractions were found in the 1.10 *d* and 1.14 *d* sucrose density regions after isopycnic centrifugation. These preparations were lighter than cell surface membranes of Hela cells[13] or liver cells[14] which were reported to have buoyant densities in sucrose between 1.16–1.18 *d*. Perdue and Sneider[7] originally noted this species difference and found that chicken fibroblasts contain relatively more lipid than mammalian cells. The lighter chick embryo fibroblast membrane fractions apparently reflect this difference in lipid composition. Our observations with the heavier fractions are also consistent with reports by Pollak and Woog[15], who showed that embryonic chicken liver mitochondria band at a sucrose density between 1.18–1.20 *d*. The absence of a visible turbid band in the 1.10 sucrose density region after labeling cells with leucine for 48 h may be due to the limiting amount of amino acids (10% of normal) used.

Perdue[16] also found that CTPase activity was associated preferentially with the plasma membrane fraction and suggested this enzyme could be used as a criterion

for plasma membrane purification[7]. Indeed, compared with the crude pellet, our data (Table I) indicated that the specific activity of CTPase was concentrated almost 4-fold in the sucrose density region of 1.10 g/ml, and compares favorably with previously published data[7] for purified plasma membranes from chick fibroblasts. The relatively high specific activity of fucose also confirmed previous observations of Atkinson and Summers[13] and Gahmberg[17] that purified plasma membranes are enriched in fucose.

Using 11% gels, the apparent molecular weight distribution of proteins and glycoproteins in chick embryo fibroblast plasma membranes resembled data reported for hamster kidney fibroblasts by Gahmberg[17]: protein subunits were broadly distributed whereas major glycoproteins appeared in the apparent molecular weight region between 82000–110000. It should be cautioned that the molecular weights noted are based on relative mobilities of marker proteins, and others[8,18,19] have noted the anomalous mobility of glycoproteins after sodium dodecyl sulfate gel electrophoresis.

Solubilized, non-delipidated membrane fractions contained a high percentage of a low molecular weight component rich in leucine, glucosamine, and fucose. In this context, Gahmberg[17] found a fast-moving glucosamine-containing peak, but was unable to observe peaks near the tracking dye when hamster fibroblasts were grown in the presence of labeled leucine or fucose. He concluded on the basis of solvent extraction studies and other evidence that the material was lipid. Other workers[20,21] have also identified this fast-moving band as a glycolipid using erythrocyte membranes from other species. Although we did not analyze this material, it is noted that Laico *et al.*[22] isolated "miniproteins" of mol. wt 5000 from a variety of membranes.

Throughout this study, solubilized preparations were dialyzed anaerobically overnight in the presence of mercaptoethanol and dithiothreitol to avoid oxidative destruction. These precautions notwithstanding, our results suggest that the irreproducible electrophoretic patterns obtained solely with fucose-labeled preparation may be due to lytic activities which render relatively low molecular weight components and labile glycoproteins dialyzable after storage: since fucose always occurs in exposed, terminal positions in oligosaccharides[23], this sugar would be lost most readily. Other workers have employed enzyme inhibitors such as fluorescein mercuric acetate[24] or iodoacetate[13] in preparing membranes: however in this initial study, we avoided using inhibitors since CTPase activity was used as a guide in membrane fractionation.

In a recent communication, Nowakowski *et al.*[25] indicated that maximal incorporation of fucose in HeLa cell plasma membranes occurs during the late S phase of the cell cycle. In view of the importance of fucose as an antigenic determinant[26], and the suggested phenotypic differences noted in fucose-rich components, future studies are planned comparing stabilized preparations derived from synchronized cultures of leukosis virus susceptible and resistant cells.

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