Carter, H. E., Hearn, W. R., Lansford, E. M., Jr., Page, A. C., Jr., Salzman, N. P., Shapiro, D., and Taylor, W. R. (1952), *J. Amer. Chem. Soc. 74*, 3704.
Carter, J. H., Du Bus, R. H., Dyer, J. R., Floyd, J. C., Rice, K. C., and Shaw, P. D. (1974), *Biochemistry 13*, 1221.
Chirpich, T. P., Zappia, V., Costilow, R. N., and Barker, H. A. (1970), *J. Biol. Chem. 245*, 1778.
Costilow, R. N., Rochovansky, O. M., and Barker, H. A. (1966), *J. Biol. Chem. 241*, 1573.
Dyer, J. R. Hayes, H. B., Miller, E. G., Jr., and Nassar, R. F. (1964), *J. Amer. Chem. Soc. 86*, 5363.
Floyd, J. C., Bertrand, J. A., and Dyer, J. R. (1968), *Chem. Commun.*, 998.
Kitagawa, T., Miura, T., Fujiwara, K., and Taniyama, H. (1972), *Chem. Pharm. Bull. 20*, 2215.
Meister, A. (1965), Biochemistry of the Amino Acids, New York, N. Y., Academic Press.
Nakanishi, K., Ito, T., and Hirata, Y. (1954), *J. Amer. Chem. Soc. 76*, 2845.
Paśś, L., and Raczynska-Bojańowska, K. (1969), *Acta Biochim. Pol. 16*, 297.
Raczyńska-Bojanowska, K., Tye, M., Paśś, L., and Kotula, Z. (1969), *Bull. Acad. Pol., Sci. Ser. Sci. Biol., 17*, 203.
Rao, S. L. N., Ramachandran, L. K., and Adiga, P. R. (1963), *Biochemistry 2*, 298.
Smissman, E. E., Sharpe, R. W., Aycock, B. F., van Temelen, E. E., and Peterson, W. H. (1953), *J. Amer. Chem. Soc. 75*, 2029.
Tam, A. H.-K., and Jordan, D. C. (1972), *J. Antibiot. 25*, 524.
van Tamelen, E. E., and Smissman, E. E. (1953), *J. Amer. Chem. Soc. 75*, 2031.
Vogel, H. J. (1959), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 18*, 345.

Polysaccharide Production by Cultured B-16 Mouse Melanoma Cells†

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ABSTRACT: Polysaccharide and glycoprotein production by two clones of the B-16 mouse melanoma and a primary explant culture of syngeneic normal iris melanocytes was studied by exposure of cultures to [^{3}H]glucosamine and $^{35}SO_4^{2-}$. Products were fractionated by differential salt extraction and porous glass bead chromatography. Neither the melanotic nor the amelanotic clones produce significant quantities of hyaluronic acid, a product which accounts for about half of the total labeled fraction for the iris melanocytes. A high molecular weight chondroitin of varying sulfate content, predominantly 4-sulfate, is a major product of the cloned lines; this polysaccharide was not detected in the iris cultures. Heparitin sulfate was present in all cells but appeared to be somewhat higher in content for the tumorigenic clones.

Cell surface properties are often markedly altered as a result of neoplastic transformation. Properties such as agglutination by plant lectins, the expression of tumor associated antigens, loss of histocompatibility antigens and variations in glycolipid and glycoprotein components have been described. We have recently reported on the alteration of mucopolysaccharide synthesis associated with virus-induced cellular transformations (Satoh *et al.*, 1973a). The present study characterizes and compares the complex polysaccharides produced by B-16 mouse melanoma cell cultures (both melanotic and amelanotic clones) and a control population of normal melanotic melanocytes obtained from syngeneic irises. A preliminary report has been presented (Satoh *et al.*, 1973c).

Materials and Methods

The origins of the B-16 melanotic melanoma cell line used in these experiments have been previously described (Kreider *et al.*, 1973). The amelanotic clone was isolated from the stock B-16 tumor maintained by the Jackson Laboratory, Bar Harbor, Maine. Clonal isolates were obtained by the glass chip method (Martin, 1973); the resulting line produces only very small amounts of melanin. All cells were routinely propagated in 16-oz prescription bottles and fed with minimum essential medium with Earle's salts, 10% heat-inactivated fetal calf serum supplemented with nonessential amino acids, sodium pyruvate, and twice the usual concentration of vitamins.

Cell pellets were periodically checked for bacterial contamination in tryptose phosphate and thioglycollate broths and for yeast contamination in Sabouraud broth. *Mycoplasma* testing was performed by both culture (House and Waddell, 1967) and autoradiography of tritiated thymidine uptake (Studzinski *et al.*, 1973).

Mouse iris melanocytes were obtained by a modification of a previously described method (Ephrussi and Temin, 1960). C57BL/6J mice, syngeneic to the B16 tumor cells, were killed by cervical dislocation following ether anesthesia. The irises were aseptically excised, and cultured as primary explants for 41 hr with 20 μCi/ml of [^{3}H]glucosamine and 20 μCi/ml of $^{35}SO_4^{2-}$.

Prescription bottles containing 10–15 million cells were incubated for 24–48 hr with the same isotopically labeled precursors at identical concentrations. After the incubation period, the media was decanted and saved and the cell monolayers were released from the glass with 0.25% EDTA in

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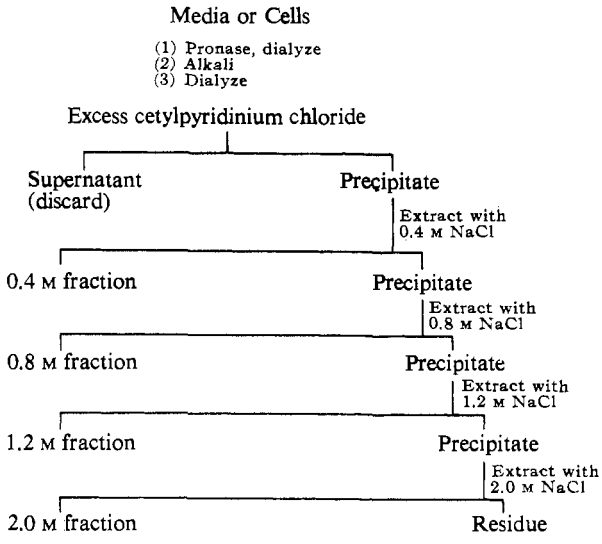

FIGURE 1: Flow chart for work-up of cell or media fractions.

calcium- and magnesium-free phosphate-buffered saline. The cell suspension was centrifuged at 600*g* for 15 min and the supernatant was discarded.

Material remaining with the pellet was identified as cell associated and the remainder as media products. These samples were analyzed separately by the following general procedure (Figure 1). After adjustment to pH 7.0, 3.75 mg each of umbilical cord hyaluronic acid and cartilage chondroitin sulfate were added as carrier followed by 2 mg/100 ml of Pronase. The resulting mixture was digested for 18 hr at 37°. After dialysis for 6 hr against 0.2 M NaCl, the resulting solution was adjusted to pH 12.5 with NaOH and allowed to stand for 18 hr at 25°. Salts and small breakdown products were removed by extensive dialysis against 0.2 M NaCl followed by distilled water. Polyanions were precipitated by addition of 100 mg of Celite and 1.5 ml of 2% cetylpyridinium chloride per 100 ml. The precipitates were harvested by centrifugation, washed with the cetylpyridinium chloride solution and fractionally eluted with 0.4, 0.8, 1.2, and 2.0 M NaCl utilizing 20 ml once followed by 10 ml four times for each extraction. The cetylpyridinium chloride was removed by dialysis at 43° against 0.03 M NaCl and H_2O and the solutions were concentrated by lyophilization. Fractionation of the individual eluates was performed by chromatography on 0.9 × 60 cm columns of CPG[1] 10–240 porous glass beads in 0.5 M $CaCl_2$. The flow rate was 24 ml/hr and 0.875-ml fractions were collected. Marker saccharides of known molecular weight were generally included and the fractions were screened for radioactivity by liquid scintillation counting and for uronic acid content by orcinol analysis (Brown, 1946). Recovery of radioactivity was invariably 95–100%. The markers employed are detailed in each figure legend. The identification of labeled amino sugar was performed following 24-hr 6 N HCl hydrolysis *in vacuo* of appropriate fractions. The hydrolysates were dried *in vacuo* and chromatographed on a Beckman 120C amino acid analyzer employing a stream-splitting attachment and utilizing [^{14}C]glycine and unlabeled glycine, glucosamine, and galactosamine as markers. As a rule, nearly quantitative recovery of tritium label was accounted for by the two amino sugars. The sole exceptions are the sialic acid containing glycopeptides present in the 0.4 M salt eluate fractions. For additional details, see Figure 2.

[1] Abbreviation used is: CPG, controlled pore glass.

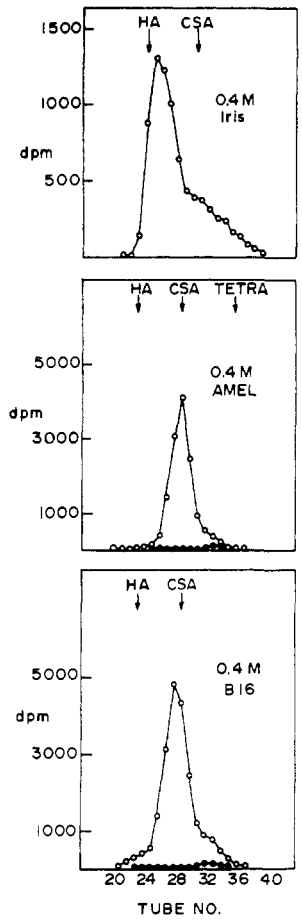

FIGURE 2: Chromatography of the 0.4 M salt eluate fraction on a 0.9 × 60 cm column of CPG 10–240 porous glass beads. The eluting solvent was 0.5 M $CaCl_2$ and each fraction contained 0.875 ml. The flow rate was maintained at 24 ml/hr using a polystaltic pump from Buchler Instruments, Inc. The marker saccharides were located by orcinol analyses and their peak positions are indicated. Blue dextran appears at tube 20–21 and monosaccharide (glucuronic acid) at tube 40. The diffusion of the markers is such that the hyaluronic acid fraction (0.5 mg) appears in 2–3 tubes and the monosaccharide in 3–4 tubes: (○) ^{3}H label; (●) ^{35}S. Fractions were combined as follows: iris I 21–28; II, 29–32; III, 33–40; Amel, I, 21–25; II, 26–30; III, 31–36; B16, I, 21–25; II, 26–30; III, 31–36.

Cellulose acetate electrophoresis was carried out in a Beckman microzone apparatus in pH 3.0 pyridine–0.1 N formic acid buffer. Strips were sectioned and radioactivity was assessed by scintillation counting; appropriate marker polysaccharides were run on parallel strips and detected by staining with Alcian Blue (Herd, 1968).

Susceptibility of individual fractions to enzymatic digestion was estimated in most cases by measuring dialyzable products following incubation with the appropriate enzyme for 18–24 hr. In several cases, fractions were rechromatographed on the CPG column following enzymatic digestion. Details are given in the appropriate figure legend.

Molecular size estimates are based on elution behavior on the glass bead columns relative to standards whose molecular weight ($\bar{M}_w$) had been independently determined by equilibrium sedimentation methods. The details of this calibration procedure will be published separately.

Testicular haluronidase was obtained from Worthington, Inc.; Pronase from Calbiochem; chondroitinase ABC and ACII from Miles Laboratories; hexosaminidase activity was present in an emulsion preparation obtained from Miles Laboratories; neuraminidase (*Vibrio cholera*) was obtained from Calbiochem; staphylococcal hyaluronidase was obtained

TABLE I: Summary of CPC-Elution Results.[a]

	0.4 M		0.8 M		1.2 M		2.0 M	
	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S
B-16	528	52	1234	332	748	188	271	78
Amelanotic	619	44	257	227	191	152	20	14
Iris melanocytes	1073	1.8	429	7.8	207	9.7	203	8.7

[a] Values are expressed in dpm $\times 10^{-3}$ per 10^6 cells except for the iris melanocytes which represent cells from 48 irises. Based on a presumed DNA content of 16 pg/cell, approximately 3.2×10^6 cells were present.

from Organon. Heparitinase and heparitin sulfate were generous gifts from Dr. Alfred Linker to whom we are greatly indebted.

Umbilical cord, vitreous humor, and skin hyaluronic acid were either obtained commercially or prepared in this laboratory. Chondrotin 4-sulfate and chondroitin tetrasaccharide were prepared in this laboratory following standard procedures (Davidson and Meyer, 1954). Radioactive substrates were obtained from New England Nuclear Corp. Scintillation counting was performed on an Intertechnique Model SL36 spectrometer using 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazole)]benzene (40:1) as scintillators and Aquasol[2] for aqueous sample counting and for electrophoresis or chromatography screening. Computation of absolute activities was done by a computer program developed from appropriate standard values.

Chemicals and solvents were the best commercially available.

Gas–liquid chromatography for determination of monosaccharide components was performed following hydrolysis in 1 N H_2SO_4 for 4 hr at 100° utilizing the alditol acetate procedure (Gunner *et al.*, 1961). A Packard 7201 chromatograph was used with a 6 ft $\times$ 1/8 in glass column of 3% ECNSS on Gas Chrom Q 100–120 mesh, operated at a temperature of 155–190° (Sloneker, 1972).

Polysaccharide identification was based on a combination of criteria which included as a minimum identification of the amino sugar, enzymatic susceptibility, and charge properties as determined by elution from the cetylpyridinium chloride precipitates.

Results

The overall incorporation figures based on the differential salt elution of the cetylpyridinium chloride precipitates are presented in Tables I and II. Although the amount of isotope incorporated into the fractions varies, this may reflect either transport or pool size parameters as well as absolute differences in synthetic rates of specific components.

0.4 M Salt Eluate. Fractionation of the 0.4 M sodium chloride eluate on CPG 10–240 glass beads produced the patterns shown in Figure 2. Two features are worthy of comment. Neither the melanotic nor the amelanotic B-16 clones exhibit any significant amount of material eluting at the position of hyaluronic acid. In addition, the apparent molecular weight of the major portion (peak II) of the 0.4 M eluate fraction is in the range of 14,000, the molecular weight of the chondroitin 4-sulfate standard employed as a marker. This figure is appreciably lower than any hyaluronic acid sample studied by us and is not likely to have arisen from enzymatic (hyaluronidase) digestion since gross size heterogeneity is absent. The electrophoretic mobility of peak II, however, was generally intermediate between that of hyaluronic acid and chondroitin sulfate, suggesting the presence of a significant number of anionic residues. Treatment of fraction II with neuraminidase followed by electrophoresis or rechromatography on CPG produced the results shown in Figures 3 and 4. As is evident from the electrophoretic and chromatographic behavior of these fractions following digestion with neuraminidase, approximately 50% of the incorporated radioactivity is released as sialic acid. The identity of the sialic acid was confirmed as follows. Following reaction with neuraminidase, the products were separated on a CPG column after the addition of standard *N*-acetylneuraminic acid carrier. The column was analyzed for radioactivity and for sialic acid by the thiobarbituric acid procedure (Aminoff, 1961). The results, shown in Figure 4, demonstrate an exact correspondence between the low molecular weight radioactive component and the standard *N*-acetylneuraminic acid. Paper chromatography of the sialic acid peak revealed the bulk (80%) of the material had an R_F identical with that of standard *N*-acetylneuraminic acid while the remainder had a mobility comparable to that of *N*-glycolylneuraminic acid (Spiro, 1960). There was insufficient material present to confirm the identity of the presumed *N*-glycolyl derivative. The radioactivity in the fast moving fraction (Figure 4), presumably predominantly glycopeptide in nature, contains tritium label in both glucosamine (6.2%) and galactosamine (93.8%) although neither appeared susceptible to hexosaminidase action following the neuraminidase treatment. Examination of the *N*-acetylneuraminic acid free glycopeptide for neutral sugar revealed the presence of mannose

TABLE II: Differential Incorporation into Cell and Media Fractions.[a]

Fraction (M)	Cells		Media	
	B-16	Iris	B-16	Iris
0.4	33.5 (4.3)	53.3 (20.6)	16.9 (14.7)	58.0 (35.5)
0.8	35.6 (4.6)	27.1 (10.5)	45.7 (39.8)	19.5 (11.9)
1.2	12.0 (1.5)	11.5 (4.5)	29.1 (25.4)	10.4 (6.4)
2.0	18.9 (2.4)	8.1 (3.1)	8.4 (7.3)	12.2 (7.5)

[a] Per cent of ^3H label found in the salt eluates. The figures in parentheses refer to the percentage of the *total* saccharide fraction (cells plus media). The proportion of label incorporated into the cell and media fractions was the same for the amelanotic clone as for the B-16 although the former showed a higher amount of isotope in the 0.4 M eluate fraction (see Table I).

[2] Commercial available xylene–Triton formula from New England Nuclear, Inc.

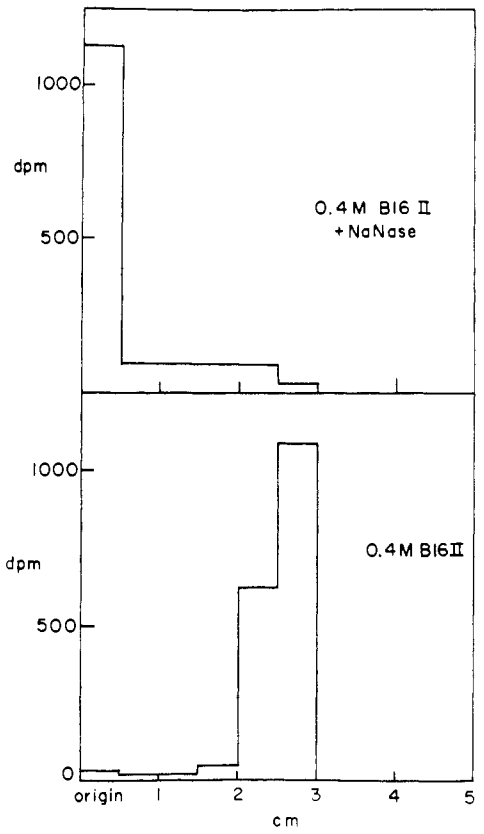

FIGURE 3: Cellulose acetate electrophoresis of B16 0.4 M salt eluate fraction II and fraction II following incubation with neuraminidase. Electrophoresis was carried out at a current of 0.22 mA/cm for 30 min. The strips were dried, sectioned into 0.5-cm pieces, and assayed for radioactivity. The positions of standards, run on parallel strips, are indicated in Figure 5.

and galactose in a ratio of 1 to 4. The galactose content was estimated to be equimolar to that of sialic acid implying that a mixture of glycopeptides is present.

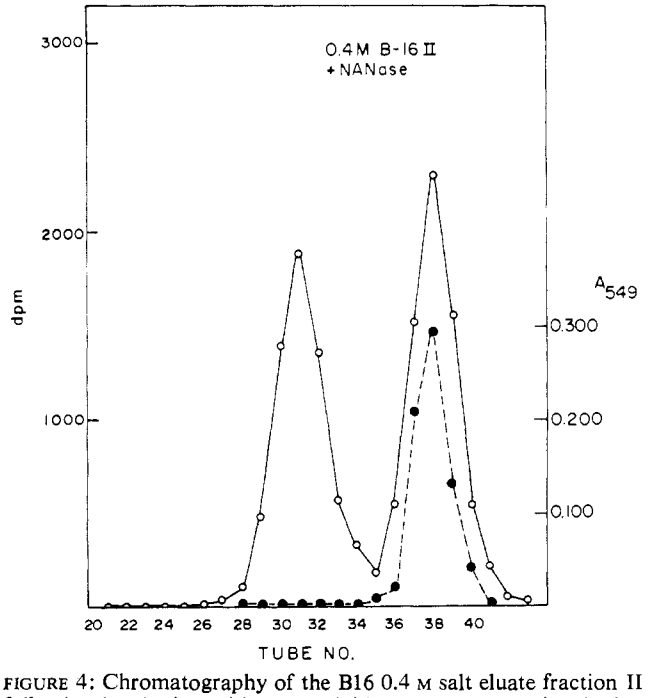

FIGURE 4: Chromatography of the B16 0.4 M salt eluate fraction II following incubation with neuraminidase. The enzyme incubation was carried out for 20 hr at 37° following which 200 μg of *N*-acetylneuraminic acid (NAN) was added prior to placing the sample on the column: (○) ^3H; (●) A_{549}.

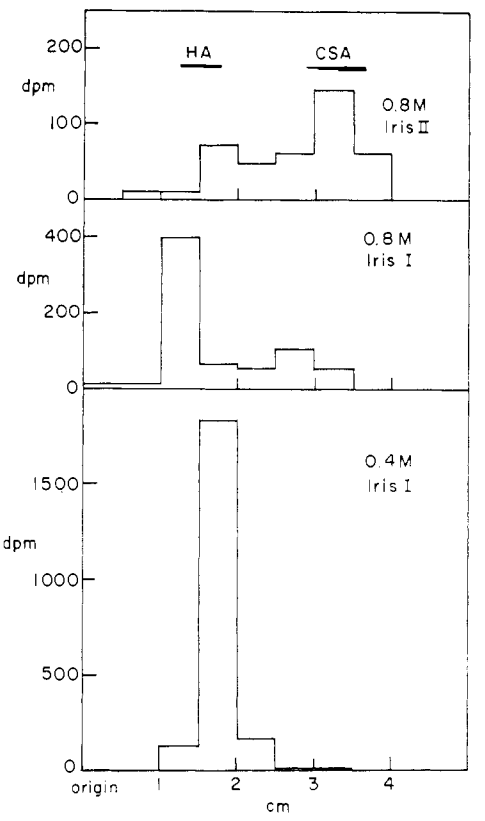

FIGURE 5: Cellulose acetate electrophoresis of the 0.4 and 0.8 M salt eluate fractions obtained from iris melanocytes. Electrophoresis was carried out for 17 min at 18 V/cm. The strips were dried, sectioned into 0.5-cm pieces, and assayed for radioactivity. The position of marker saccharides on parallel control strips is indicated.

Treatment of the 0.4 M eluate fraction II derived from the melanotic cell culture media with bacterial hyaluronidase followed by chromatography on the CPG column revealed that approximately 7% of this fraction was susceptible to the bacterial hyaluronidase. This apparently represents primarily an undersulfated chondroitin sulfate of molecular weight 13,000–15,000 since galactosamine is the predominant amino sugar present and cleavage to oligosaccharides took place. The small amount of ^{35}S label originally found in peak II apparently remains associated with this fraction. The materials obtained from the amelanotic cell culture were virtually identical with those of the B-16, although present in larger relative amounts.

In marked contrast to these tumorigenic clones, the 0.4 M salt eluate obtained from the normal iris melanocytes showed a major peak eluting near hyaluronic acid with trailing areas of lower molecular weight material. The major fraction (peak I) was identified as hyaluronic acid base on its complete susceptibility to bacterial hyaluronidase, essentially exclusive content of glucosamine, cetylpyridinium chloride elution properties, electrophoretic behavior (Figure 5), and molecular weight range. The lower molecular weight fraction, peak III, contained predominantly glucosamine and apparently represents both glycopeptide fragments and possibly an undersulfated heparitin sulfate since it was not significantly susceptible to testicular hyaluronidase or chondroitinase and had an electrophoretic mobility characteristic of sialic acid containing components. A summary of the properties of the 0.4 M salt eluates is given in Table III.

0.8 M Salt Eluate. Chromatography of the 0.8 M salt eluate fractions is illustrated in Figure 6. In all cases, two clearly resolved peaks were obtained. The faster moving eluted near

TABLE III: Properties of the 0.4 M NaCl Fraction.[a]

	% of Total	Glu-NH_2[b]	Gal-NH_2[b]	Sialic Acid	Heparitin	Hyaluronic Acid	Chondroitin
B-16							
I	0.97	6	94			<0.1	
II	11.1	2.9	47.1	50			
III	2.6	63.6	20.6	15.8	1.57		
Amel							
I	0.81					0.48	0.22
II	35.4	3.4	46.6	50			
III	10.3	27.6	35	37.4	2.44		
Iris							
I	36.9	100	0	0		36.9	
II	10.7	87.1	12.9	0		7.4	
III	8.5	63	26	11	1.1	2.6	

[a] Composition of the 0.4 M salt eluate fractions. Per cent of total refers to the percentage of incorporated radioactivity relative to the total labeled saccharide fraction. The figures for the amino sugars and sialic acid are percentages of label within the specific peak. The sialic acid content was calculated from neuraminidase-sensitive radioactivity following resolution of released sialic acid from glycopeptides on the CPG column. Polysaccharide values are as percentages relative to the total labeled saccharide fraction. Heparitin refers to dialyzable radioactivity following incubation for 18 hr with heparitinase. Chondroitin refers to chondroitinase ABC susceptible material, presumably of very low sulfate content. [b] $GluNH_2$ = 2-amino-2-deoxy-D-glucose; $GalNH_2$ = 2-amino-2-deoxy-D-galactose.

the void volume of the column and nearly coincident with a standard sample of umbilical cord hyaluronic acid; the slower moving fraction emerged just behind chondroitin 4-sulfate. Both the melanotic and amelanotic B-16 clones showed significant sulfate incorporation in both peaks whereas ^{35}S was apparently completely absent in the higher molecular weight material from the iris melanocytes and present in only trace amounts in the second peak. The latter appeared to have a comparable molecular weight range to the corresponding fraction of the tumorigenic lines.

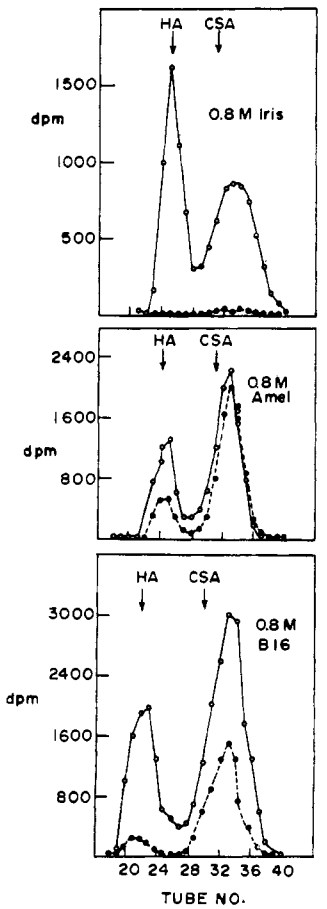

FIGURE 6: Chromatography of the 0.8 M salt eluate fraction. Conditions were as described for Figure 2: (○) ^{3}H; (●) ^{35}S. Fractions were combined as follows: iris, I, 21–28; II, 29–40; Amel, I, 21–27; II, 29–36; B16, I, 19–26; II, 27–38.

The amino sugar present in peak I of both the melanotic and amelanotic clones was almost exclusively galactosamine whereas primarily glucosamine (98.8%) was present in the melanocyte fraction. Treatment of the melanotic peak I material with bacterial hyaluronidase and rechromatography on the glass bead column produced the results shown in Figure 7. The nearly complete susceptibility of this fraction to bacterial

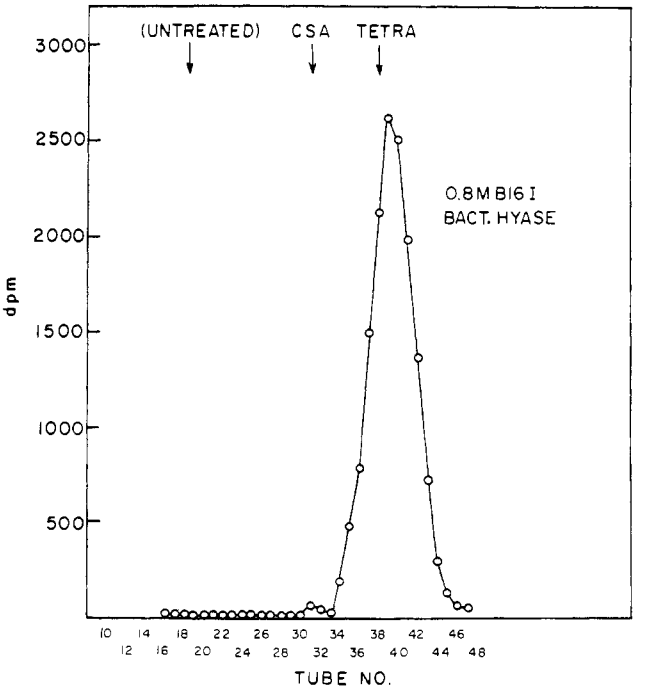

FIGURE 7: Chromatography of the B16 0.8 M salt eluate fraction peak I following 18-hr incubation with bacterial hyaluronidase: (○) ^{3}H.

TABLE IV: Properties of 0.8 M NaCl Fraction.[a]

	Mol Wt	Glu-NH_2	Gal-NH_2	Hyaluronidase	Heparitinase	Chondroitinase
B-16						
Peak I	35,000+	0	100	100		99.8
Peak II	10,000–14,000	89.7	10.3		84	20
Amelanotic						
Peak I	35,000+	5.5	94.5	100		
Peak II	10,000–14,000	93.5	6.5			
Iris						
Peak I	35,000+	90.8	9.2	86.4	<0.1	
Peak II	10,000–14,000	72.4	27.6		63.1	48.3

[a] Summary of analytical data for the 0.8 M salt eluate fractions. The molecular weight values are based on the behavior of the labeled components relative to standard samples during chromatography on the CPG column. The 35,000 figure is regarded as minimal since the fraction overlaps slightly with blue dextran. The amino sugar values are as percentages of tritium label found for glucosamine and galactosamine. The figures for hyaluronidase, heparitinase, and chondroitinase refer to percentage of tritium label rendered dialyzable following 18-hr incubation with bacterial hyaluronidase, heparitinase, or chondroitinase ABC.

hyaluronidase suggests a structure analogous to that of chondroitin (Davidson and Meyer, 1955). The approximate molecular weight of the breakdown products, in the range of di- to tetrasaccharide, also shows that the degree of sulfation is 50% or less and that the sulfate groups must be randomly distributed. Were this not the case, a core of higher molecular weight containing the bulk of the sulfate would be present. The amelanotic clone contained an identical component in similar relative amounts. The high molecular weight fraction obtained from the iris melanocytes was identified as hyaluronic acid based on enzymatic susceptibility, amino sugar content, and electrophoretic mobility (Figure 5).

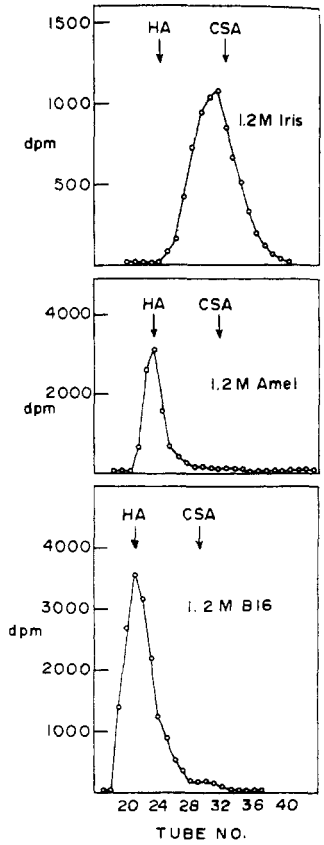

FIGURE 8: Chromatography of the 1.2 M salt eluate fraction. Conditions were as described for Figure 2: (O) ^3H. Fractions were combinded as follows: iris, I, 24–38; Amel, I, 20–25; II, 26–33; B16, I, 19–26; II, 28–34.

The lower molecular weight fraction (peak II) isolated from both the melanotic and the amelanotic clones was identified as predominantly heparitin sulfate admixed with a small amount of slightly undersulfated chondroitin sulfate which had a molecular weight comparable to that of cartilage chondroitin 4-sulfate but appreciably lower than the chondroitin-like peak I. These two fractions could not be resolved by chromatographic or electrophoretic means and their relative proportions were estimated from a composite of amino sugar content and enzymatic susceptibility data (Table IV). The composition of the peak II iris melanocyte fraction was similar to that of the melanotic clone. Data are summarized in Table IV.

1.2 M Salt Eluate Fraction. The chromatographic results of the 1.2 M fractions are illustrated in Figure 8. For both the melanotic and the amelanotic clones, almost the entire fraction is represented by a nearly homogeneous relatively high molecular weight, galactosamine-containing, chondroitinase-susceptible component. Based on digestion with bacterial hyaluronidase, the degree of sulfation is better than 95% (approximately 1 bond in 20 was susceptible); a trace amount of heparitin sulfate was present. In contrast to this, the iris melanocyte material appears to be of lower molecular weight and is a mixture of chondroitin sulfate and heparitin sulfate in a ratio of approximately 3:1. Data for this fraction are summarized in Table V.

2.0 M Salt Eluate Fraction. This fraction was substantially the same as the 1.2 M material for all samples. There was glucosamine present in both fractions, amount to 10% of the total in this fraction. The ratio of ^{35}S to ^3H incorporation was slightly higher in this fraction than in the 1.2 M eluate but electrophoretic examination of the fractions provided no evidence for significant oversulfation of the chondroitin chain. In both the 1.2 and 2.0 M salt eluate fractions, the iris material appeared to have a much broader molecular weight distribution, possibly due to the mixing of cell associated and media fractions. Insufficient material was available to permit molecular weight estimates by reliable alternative methodology. Data are given in Table VI.

A summary of the fractionation and identification data for the melanotic clone and iris melanocyte material is presented in Table VII.

TABLE V: Properties of 1.2 M NaCl Fractions.[a]

	Mol Wt	Glu-NH_2	Gal-NH_2	Hyaluronidase	Heparitinase	Chondroitinase
B-16						
I	35,000+	0	100	Slight		99
II	10,000–14,000	54.4	45.6		34.8	52.8
Amelanotic						
I	35,000+	0	100			
II	10,000–14,000	17.1	82.9			
Iris						
I	10,000–16,000	24.1	75.9		15.2	65.7

[a] Summary of analytical data for 1.2 M salt eluate fractions. Other details are as described for Table IV.

TABLE VI: Properties of 2.0 M NaCl Fractions.[a]

	Glu-NH_2	Gal-NH_2	Hyaluron-idase	Hepari-tinase	Chondroi-tinase
B-16					
I	1.2	98.8	81.8		98.1
II	63.0	37.0		18.8	
Iris					
I	5.3	94.7			83.2
II	16.5	83.5			

[a] Summary of analytical data for the 2.0 M salt eluate fractions. The material from the amelanotic clone amounted to less than 1% of the total incorporated radioactivity and was not further analyzed. The hyaluronidase employed was of testicular origin (Worthington). Other conditions were as described for Table IV.

Discussion

There are several unusual properties of the proteoglycans and glycoproteins in cultured melanoma cells. These include the apparent absence of any significant quantity of hyaluronic acid, the relatively high proportion of heparitin sulfate and the presence of a relatively high molecular weight chondroitin-like molecule with varying levels of sulfation. In addition, the glycopeptide fraction, appearing in the 0.4 M salt eluate, is distinctly different in the melanotic clone as compared to the iris melanocytes.

The ideal control cell to compare with the B-16 melanoma cell would be a cutaneous melanocyte, the presumed cell from which this neoplasm originally derived in the C57BL/6J mouse strain. These normal cells are virtually impossible to culture in pure form in sufficient numbers for these experiments. Accordingly, we studied the next most suitable control cell, the pigmented melanocytes of the C57BL/6J iris. Although these cells are possibly of a different embryologic origin, they are morphologically and functionally identical with the cutaneous melanocytes. In addition, the iris is relatively free of other cell types and the cells appeared to retain phenotypic characteristics during the brief tenure of the experiment. The polysaccharide products of the iris cells were in no way unusual and rather similar to those observed for other somatic cells (Abbott *et al.*, 1971). These consisted of hyaluronic acid, chondroitin (4) 6-sulfate, and a small amount of heparitin sulfate. Neither dermatan sulfate nor keratan sulfate was detected although quantities less than 0.5% of the total polysaccharide fraction would probably not have been found especially if these macromolecules were synthesized at a significantly slower rate than those proteoglycans actually identified.

Hyaluronic acid, normally the major component of the 0.4 M salt eluate, and a predominant product of the iris melanocyte was not detected in the melanotic B-16 clone and was present to the extent of less than 0.5% in the amelanotic line. A major radioactive component of this fraction was sialic acid, all of which appeared susceptible to neuraminidase action; some *N*-glycolylneuraminic acid may be present. Although none of the radioactivity in the 0.4 M fraction partitioned into the organic phase following extraction with chloroform–methanol, the presence of glycolipid in this fraction was not examined and some of the sialic acid may be associated with gangliosides or similar components. The glycopeptides may be derived from plasma membrane or may represent products secreted by the cells. As is evident from the data presented in Table VII, under the experimental conditions employed, the majority of the incorporated radioactivity appears in the

TABLE VII: Summary of Incorporation Data.[a]

	Cell Associated	Media	Glycopeptide		Hyaluronic Acid	Chondroitin (High Mol Wt)	Chondroitin Sulfate	Heparitin Sulfate
			Sialo	Asialo				
B-16	12.8	87.2	5.96	5.32	<1.0	44.0	6.0	22.3
Amelanotic	14.1	85.9	21.6	18.0	<1.0	23.5	3.0	16.6
Iris melanocytes			1.0	2.2	53.2	<1.0	22.2	11.2

[a] The relative amounts of tritium label incorporated into media and cell associated fractions are given for the two tumorigenic clones; the iris melanocytes were not separated into cell and media fractions. The proportions of the various polysaccharides are given in percentages and represent a composite of analytical data as described in the text. The B-16 and amelanotic clone data are for the media polysaccharides only.

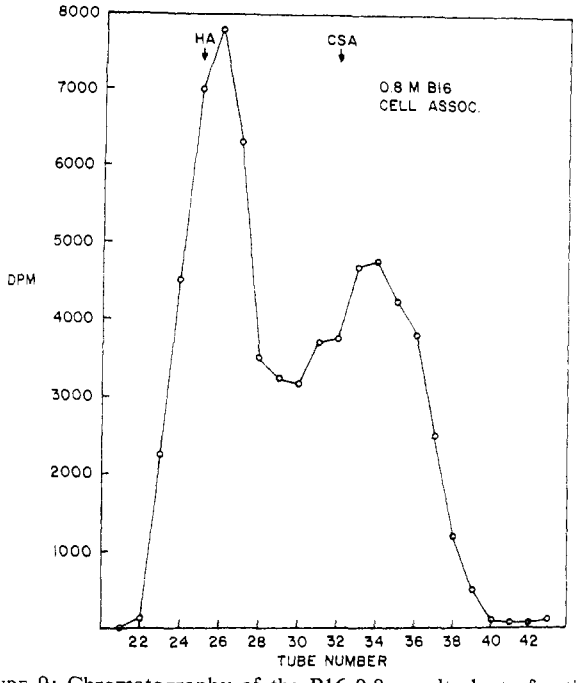

FIGURE 9: Chromatography of the B16 0.8 M salt eluate fraction obtained from cell-associated material: (○) ^{3}H.

medium. However, several of the cell-associated fractions were examined in detail to ascertain if their proteoglycan components mirrored those observed in the medium. In general, the fractionation properties on the CPG columns were similar (Figure 9) with some suggestion of a broader molecular size distribution apparent in the cell-associated material.

Approximately 50% of the incorporated radioactivity of the 0.4 M salt eluate fraction, peak II, was found in sialic acid. The remainder was distributed between the amino sugars with galactosamine predominant. Following removal of the sialic acid, the electrophoretic mobility was nil suggesting the absence of significant amounts of uronic acid containing saccharides. The 0.4 M salt fraction (peak II) contained a small amount of material which was bacterial hyaluronidase susceptible. Since the amino sugar present in this fraction was identified as galactosamine, it seems likely that this material represents a small amount of low-sulfated chondroitin sulfate of molecular weight range 13,000–15,000. The molecular weight of the 0.4 M salt fraction ranges from 2,000 to 15,000 with the bulk of the material appearing at about 14,000. This value seems high for most glycopeptide fragments since the saccharide chains of glycoproteins rarely exceed ten monomer units. However, there is no information about the parent structures from which these fragments are derived. It is clear, however, that the bulk of this fraction is glycopeptide in nature for the B-16 lines. A recent study has described the absence of hyaluronic acid synthesis by mouse mastocytoma cells grown in suspension culture (Lewis *et al.*, 1973). However, the overall synthesis of proteoglycan was low in this system and may be related to the failure of these cells to attach to the glass substrate since similar observations relative to proteoglycan synthesis have been made for normal chondrocytes which spontaneously detach from their glass substrate and grow as floaters (Abbott *et al.*, 1971). It should be noted that the absence of hyaluronic acid production by neoplastic lines is unusual since several virus-transformed tumorigenic lines display a marked increase in the production of this proteoglycan (Satoh *et al.*, 1973a).

The main component of the 0.8 M salt eluate fraction of the melanoma cells appeared to be a relatively high molecular weight polysaccharide containing only galactosamine as the amino sugar component. This material was susceptible to bacterial hyaluronidase yielding breakdown products which were approximately di- or tetrasaccharide. In addition, the fraction was completely susceptible to chondroitinase ABC. Since the mode of action of bacterial hyaluronidase is such that hexosaminidic bond cleavage can occur only at non-sulfated loci, it is estimated that the maximal sulfate to hexosamine ratio of the peak I polysaccharide is 0.5 and that the sulfate groups are randomly distributed. The molecular size of this material is comparable to that of umbilical cord hyaluronic acid based on their exclusion behavior on the CPG columns. Independent molecular weight ($\bar{M}_w$) measurement by sedimentation equilibrium of the hyaluronic acid standard employed gave a value of 40,000. If it can be assumed that the conformational properties of the standard and unknown are similar, then the $\bar{M}_w$ of the peak I fraction should be in this range. This assumption seems reasonable since they are both linear structures containing glycosidic linkages of identical configuration and position and there is sufficient supporting electrolyte ($\mu = 1.5$) present to prevent significant chain expansion due to electrostatic repulsion of the sulfate ester groupings. This molecular weight range is appreciably higher than that of costal cartilage chondroitin 4-sulfate (Woodward *et al.*, 1972) or of the chondroitin sulfate present in the iris melanocyte material. It is not known at this time whether the macrostructure of this proteoglycan is comparable to that of other chondroitin sulfates wherein there are multiple saccharide chains attached to a single polypeptide core or whether it more closely resembles that of hyaluronic acid which apparently is assembled as a single polysaccharide chain with little or no covalently attached protein. Chondroitinase digestion followed by paper chromatography indicated that the sulfate ester group was predominantly in the 4 position of the amino sugar.

The second component of the 0.8 M salt eluate fraction was a mixture of heparitin sulfate and a small amount of a chondroitinase-susceptible component, apparently an undersulfated chondroitin sulfate. The identification of the heparitin sulfate was based on the fact that the chondroitinase resistant portion of peak II contained only glucosamine, was completely digested by heparitinase and had a molecular weight of approximately 10,500, comparable to that of a heparitin sulfate standard. The proportion of *N*- to *O*-sulfate is not known.

The major component of both the 1.2 and 2.0 M salt eluate fraction appears to be the more fully sulfated analog of the high molecular weight chondroitin. In contrast, the iris melanocyte fraction, although also containing predominantly galactosamine, elutes at the position of the cartilage chondroitin as sulfate standard ($\bar{M}_w$ 14,000) and contains less than 1% of the high molecular weight material seen in the B-16 lines.

The polysaccharides and glycoproteins made by the melanotic and amelanotic B-16 clones appear essentially identical, although they differ somewhat in relative amounts, so it appears unlikely that tyrosinase activation and the process of melanogenesis are in anyway related to those cell surface properties defined by the components studied.

Although it cannot be definitively stated that the observed incorporation data directly measure the composition of cell-associated proteoglycans, the fact that there are common precursors for all of this group of macromolecules (UDP-glucuronic acid, UDP-*N*-acetylhexosamine) suggests that the features observed are representative and not simply reflections

of transport of pool-size phenomena. In common with several other tumorigenic lines, a significant proportion of the total incorporated radioactivity for the B-16 lines appeared in heparitin sulfate (Satoh *et al.*, 1973b). The biological role of this macromolecule remains unknown but there does not appear to be a clear distinction between it and the chondroitin-like molecules in terms of cell association. A recent report has described a relationship between heparitin sulfate and the cell cycle (Kraemer, 1972) but did not establish a biologically related difference between it and other proteoglycans or glycoproteins.

A number of investigators have noted that cell surface moieties are dependent upon culture conditions especially cell population density (Hakamori and Murakami, 1968). Cultured cells frequently fail to exhibit the same phenotype as the *in vivo* parent cell type. Although the B-16 line readily gives rise to solid tumors when inoculated into an appropriate host animal, these contain connective tissue elements, inflammatory cells, blood vessels, and the products of cell necrosis. Accordingly, definitive characterization of comparable products from solid tumor material is difficult. Studies on solid tumor material as well as the effects of population density will be the subject of a separate report.

It is tempting to speculate that the chondroitin-like molecule observed in the B-16 cell represents a replacement for the hyaluronic acid normally synthesized by the melanocyte. The organization of this polysaccharide in terms of its association with protein has not as yet been defined partly due to the limited amounts of material available from cell culture experiments. The distribution of sulfate ester groups in the 0.8 M peak I fraction appears to be about one per tetrasaccharide unit. If it can be reasoned that this fraction is the precursor of the fully sulfated analogs appearing in the 1.2 and 2.0 M salt eluates, then it seems likely that sulfation occurs subsequent to chain assembly. The mechanism or stage at which sulfation occurs is still not clearly defined and contrary proposals have been advanced by our laboratory (Davidson and Meyer, 1955; Derge and Davidson, 1972) and others (Kleine *et al.*, 1968). Based on uronic acid analyses, estimates of the amount of material present in the 0.8 M peak I fraction give values which are lower than the amount reflected by isotope incorporation data alone. Whether this reflects loss to the medium due to improper binding to the appropriate membrane fraction is unknown. It is also not clear whether the appearance of this polysaccharide reflects a loss of specificity in the hyaluronic acid synthetic apparatus (not likely) or the acquisition of a new synthetic capacity on the part of the B-16 cell line.

Although relatively small amounts of labeled material are cell associated under the experimental conditions employed, separate analysis of these fractions revealed that they contained the same type of products as those appearing in the medium with some suggestions of a broader molecular weight distribution possibly reflecting incomplete assembly. The nature of cell association remains unknown although it appears that the primary site of interaction is the plasma membrane.

References

Abbott, J., Davidson, E. A., and Holtzer, H. (1971), *Anat. Rec. 169*, 265.

Aminoff, D. (1961), *Biochem. J. 81*, 384.

Brown, A. H. (1946), *Arch. Biochem. 11*, 269.

Davidson, E. A., and Meyer, K. (1954), *J. Amer. Chem. Soc. 76*, 5686.

Davidson, E. A., and Meyer, K. (1955), *J. Biol. Chem. 211*, 605.

Derge, J. G., and Davidson, E. A. (1972), *Biochem. J. 126*, 217.

Ephrussi, B., and Temin, H. (1960), *Virology 11*, 547.

Gunner, S. W., Jones, J. K. N., and Perry, M. B. (1961), *Chem. Ind. (London)*, 255.

Hakamori, S., and Murakami, W. T. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 254.

Herd, J. K. (1968), *Anal. Biochem. 23*, 117.

House, W., and Waddell, A. (1967), *J. Pathol. Bacteriol. 93*, 125.

Kleine, T. O., Kirsig, H. J., and Hilz, H. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1037.

Kraemer, P. M. (1972), *J. Cell Biol. 55*, 713.

Kreider, J. W., Rosenthal, M., and Lengle, N. (1973), *J. Nat. Can. Inst. 50*, 555.

Lewis, R. G., Spencer, A. F., and Silbert, J. E. (1973), *Biochem. J. 134*, 455.

Martin, G. M. (1973), *in* Tissue Culture Methods and Applications, Kruse, P. F., Jr., and Patterson, M. K., Ed., New York, N. Y., Academic Press, p 264.

Satoh, C., Duff, R., Rapp, F., and Davidson, E. A. (1973a), *Proc. Nat. Acad. Sci. U. S. 70*, 54.

Satoh, C., Duff, R., Rapp, F., and Davidson, E. A. (1973b), *Proc. 9th Intern. Congr. Biochem.*, 428.

Satoh, C., Kreider, J. W., and Davidson, E. A. (1973c), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 2604.

Sloneker, J. H. (1972), *Methods Carbohydr. Chem. 6*, 20.

Spiro, R. G. (1960), *J. Biol. Chem. 235*, 2860.

Studzinski, G. P., Gierthy, J. F., and Cholon, J. J. (1973), *In Vitro 8*, 466.

Woodward, C. B., Hranisavljevic, J., and Davidson, E. A. (1972), *Biochemistry 11*, 1168.