A Comparative Study of Glycoproteins from the Surface of Control and Rous Sarcoma Virus Transformed Hamster Cells*

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ABSTRACT: Comparative studies of the glycopeptides from the surface of BHK_{21}/C_{13} cells before and after transformation by Rous sarcoma virus revealed a major difference. Cells were grown in the presence of radioactive L-fucose or D-glucosamine.

The cells were trypsinized and the material removed from the cell surface was digested with pronase. The resulting glycopeptides were cochromatographed on Sephadex G-50. The material from the transformed cells contained an enrichment of glycopeptides of apparently higher molecular weight than the major group of glycopeptides from the control cells. Control BHK_{21}/C_{13} cells also contained material of similar size to the higher molecular weight glycopeptides of the transformed cells, but in much smaller quantities. These differences were also seen when purified surface membranes isolated from control and transformed cells were dissolved in detergent, treated with pronase, and fractionated on Sephadex G-50.

Several groups have reported alterations of cells either spontaneously transformed or transformed by DNA tumor viruses (Inbar and Sachs, 1969; Burger, 1968; Wu *et al.*, 1969; Meezan *et al.*, 1969; Bosmann *et al.*, 1968; Hakomori *et al.*, 1968; Mora *et al.*, 1969). However, there are as yet no comparative studies of glycoproteins from the surface of morphologically normal cells and cells transformed by RNA tumor viruses. In light of the possible importance of these viruses in human malignancy (Heubner and Todaro, 1969), we have begun a systematic comparison of various cell lines transformed by RNA tumor viruses.

The cells used in this study were baby hamster kidney fibroblasts, BHK_{21}/C_{13}, and two clones derived from this line of cells, one transformed by the Bryan strain of Rous sarcoma virus (RSV)[1] and the other transformed by the Schmidt–Ruppin strain. Descriptions of these cell lines have been published (Macpherson and Stoker, 1962; Macpherson, 1965; Jarrett and Macpherson, 1968). The cells were maintained as monolayer cultures which were removed from the glass with trypsin. Several investigators have found that exposing intact cells to proteolytic enzymes such as trypsin (Langley and Ambrose, 1964; Winzler, 1969; Kornfeld and Kornfeld, 1969) or papain (Shimada and Nathenson, 1969; Walborg *et al.*, 1969), has resulted in the release of certain glycopeptides. Therefore, in this study of the cell surface we compared three fractions from the BHK_{21}/C_{13} cell lines: (1) the material removed from the cell surface by trypsin digestion ("trypsinates"), (2) the material recovered from the cells after the washing procedure which followed trypsinization ("washes"), and (3) purified surface membranes isolated from the trypsinized cells.

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[1] Abbreviations used are: TBS, 0.15 M NaCl–0.02 M Tris-HCl (pH 7.5); SDS, sodium dodecyl sulfate, RSV, Rous sarcoma virus.

Materials and Methods

Cell Culture. The cells used in these experiments were from early passage stocks of morphologically normal hamster fibroblasts, BKH_{21}/C_{13}; BHK_{21}/C_{13} cells transformed with the Bryan strain of RSV, C_{13}/B_4; and BHK_{21}/C_{13} cells transformed with the Schmidt–Ruppin strain of RSV, C_{13}/SR_7. Stocks were frozen so that no experiments were performed on cells passaged more than 12 times (maximum of 120 generations) in this laboratory. The cells were grown in Eagles minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% tryptose phosphate broth (Difco), 5% fetal calf serum (Microbiological Associates, Rockville, Md.), penicillin (50 units/ml), streptomycin (100 μg/ml), and anti-PPLO agent (Grand Island Biological Co.). Tests were routinely performed for the presence of *Mycoplasma* and were negative.

For all experiments, the cells were cultured in disposable roller bottles (growth area, 654.51 cm^2; Bellco Glass Co., Vineland, N. J.) which were rotated on a Bellco roller apparatus. Each bottle was inoculated with 50 ml of medium containing a total of 2×10^7 cells. Isotope was added at this time. Fifty milliliters of fresh medium without isotope was added daily to each bottle. Unless otherwise stated, cells were harvested 72–78 hr after initial transfer. In this way, all cells were exposed to isotope during the time of most rapid growth. During this time the number of cells increased from 2×10^7 cells per bottle to between 0.9×10^8 and 1.2×10^8 cells per bottle.

Trypsinization. Suspensions of single cells from cultures in essentially the same phase of growth were obtained by the following procedure. The medium was poured from each bottle, and the cells adhering to the glass were rinsed five times with 100 ml of TBS at room temperature. Ten milliliters of trypsin (1 mg/ml in TBS) was added to each bottle and the bottle was incubated in the roller apparatus for 15 min at 37°. At the end of 15 min, 10 mg of soybean trypsin inhibitor was added to each bottle. The cells were immediately cooled in an ice bath and centrifuged at 800*g* for 5 min. The supernatant

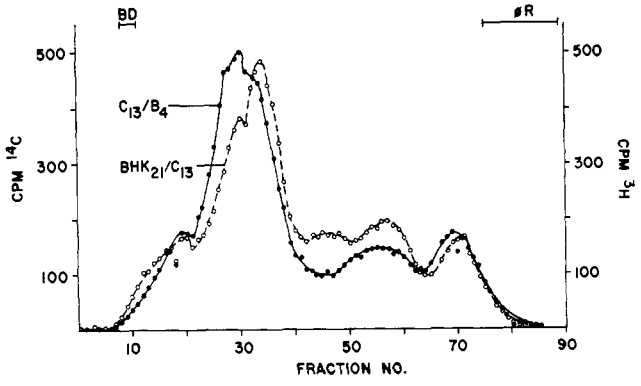

FIGURE 1: Cochromatography on Sephadex G-200 of trypsinates from C_{13}/B_4 and BHK_{21}/C_{13} cells grown in the presence of radioactive L-fucose. Cells were grown on roller bottles as described in Materials and Methods in the presence of 50 μCi of L-[^3H]fucose (C_{13}/B_4) or 25 μCi of L-[^{14}C]fucose (BHK_{21}/C_{13}) for 72 hr. Trypsinates were prepared (see Materials and Methods) and pooled just prior to chromatography on Sephadex. Columns (0.8 × 100 cm) were equilibrated and developed with 0.1 M Tris-acetate (pH 9.0), 0.1% SDS, 0.1% mercaptoethanol, and 0.01% EDTA. Samples of 0.7 ml were collected at a rate of approximately 3 ml/hr. BD represents the samples in which the Blue Dextran 2000 was eluted; ϕR represents the samples into which the phenol red marker was eluted. Profiles of L-[^3H]fucose-labeled trypsinate from C_{13}/B_4 cells (●—●), and of L-[^{14}C]fucose-labeled trypsinate from BHK_{21}/C_{13} control cells (○---○).

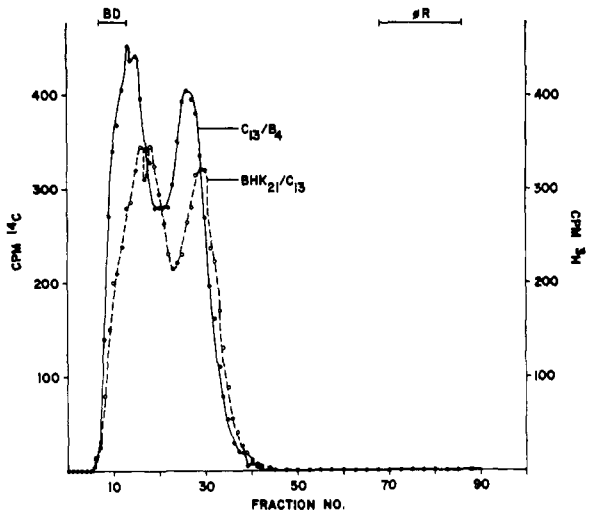

FIGURE 2: Cochromatography on Sephadex G-200 of purified surface membranes from C_{13}/B_4 and BHK_{21}/C_{13} cells grown in the presence of radioactive L-fucose. C_{13}/B_4 and BHK_{21}/C_{13} cells were grown in the presence of 50 μCi of L-[^3H]fucose and 25 μCi of L-[^{14}C]fucose, respectively, as described in Figure 1. Cells were harvested and membranes prepared as described in Materials and Methods. Membranes were dissolved in 0.1% SDS and pooled prior to chromatography. Sample size, flow rate, column size, and elution buffer were as stated in Figure 1. BD, samples in which Blue Dextran 2000 was eluted; ϕR samples in which phenol red was eluted. Profiles of L-[^3H]fucose-labeled membranes from C_{13}/B_4 cells (●—●) and of L-[^{14}C]fucose-labeled membranes from BHK_{21}/C_{13} control cells (○---○).

solutions (trypsinates) were saved and the cells washed three times in 20 ml of cold 0.16 M NaCl. The washes were combined and saved for further study. Less than 2% of the radioactivity released by trypsinization was soluble in ether. Less than 10% of the cells prepared in this manner were stainable by trypan blue.

Preparation of Surface Membranes. The cells harvested and washed as described above were suspended in 0.16 M NaCl to a concentration of 5×10^7 cells per ml. Surface membranes were prepared from these cells by the zinc ion procedure (Warren and Glick, 1969) and were counted in a hemocytometer. The purified surface membranes were stored as pellets at −20°. The pellets were dissolved in 0.1% SDS prior to fractionation.

Preparation of Trypsinates and Washes. The trypsinates and the washes from 1 to 3×10^8 cells (three roller bottles) were centrifuged at 49,000g for 20 minutes. The supernatant solutions were then lyophilized and subsequently dissolved in 2–3 ml of distilled H_2O and stored at −20°.

Pronase Digestion. Pronase digestion of trypsinates and washes was performed as described by Spiro (1965). The surface membranes were dissolved in 0.1% SDS prior to digestion with pronase. All digestions were carried out at 37° for 5 days in the presence of toluene. Fresh pronase was added daily. Digests were stored frozen at −90° until fractionated. The various combinations of trypsinates, washes, and membranes were pooled prior to pronase digestion.

Gel Filtration. Columns of Sephadex G-200 or G-50 fine (Pharmacia) were equilibrated and eluted with 0.1 M Tris-acetate buffer (pH 9.0), 0.1% SDS, 0.01% EDTA, and 0.1% mercaptoethanol. All columns measured 0.8 × 100 cm. Samples up to 0.7 ml in elution buffer were placed on the columns. Blue Dextran 2000 (Pharmacia) and phenol red were included in each sample as high and low molecular weight markers, respectively. Fractions of 0.7 ml were collected at a rate of 13 ml/hr (Sephadex G-50) or 3 ml/hr (Sephadex G-200) and prepared for scintillation counting as described below.

Preparation of Samples for Scintillation Counting. All fractions were dried in an oven at 120° and subsequently dissolved in 0.1 ml of 0.1 N NaOH. NCS (0.7 ml) obtained from Amersham–Searle, Des Plaines, Ill., and 10 ml of toluene–liquifluor (Pilot Instrument Co., Watertown, Mass.) were added to each sample. Samples were counted in a Packard Tri-Carb liquid scintillation counter for both ^{14}C and ^{3}H.

Chemical Assays. Sialic acid was determined by the thiobarbituric acid assay (Warren, 1959) after elution of the hydrolysate (0.1 N H_2SO_4, 190°, 1 hr) from Dowex (Ac) (Svennerholm, 1958). Crystalline *N*-acetylneuraminic acid was used as a standard. Fucose was determined by gas–liquid chromatography as the alditol acetate by the method of Lehnhardt and Winzler (1968). Proteins were determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard.

To determine the amount of radioactive fucose which remained as fucose, an aliquot of trypsinate obtained from BHK_{21}/C_{13} grown in the presence of L-[^3H]fucose was mixed with an aliquot of trypsinate from C_{13}/B_4 grown in the presence of L-[^{14}C]fucose. The mixture was digested with pronase and precipitated with 5% trichloroacetic acid. The supernatant solution was dialyzed 15 hr against distilled water, lyophilized, and hydrolyzed on Dowex 50 (H^+) in the presence of 0.02 N HCl for 40 hr at 100°. The hydrolysate was washed from the

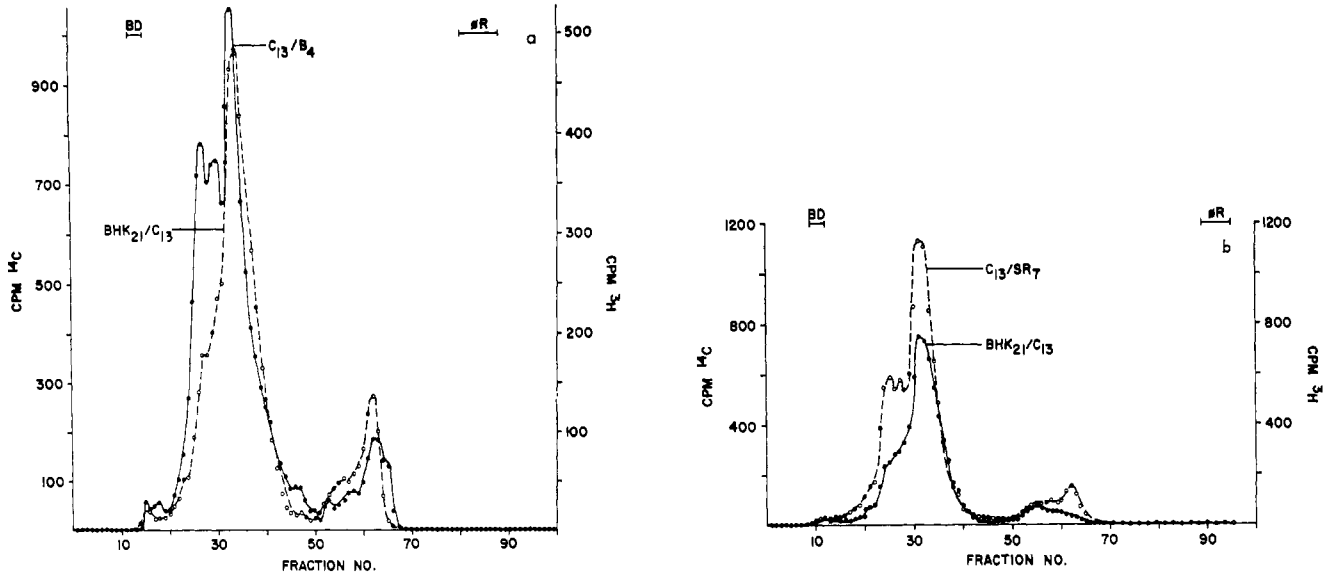

FIGURE 3: Cochromatography on Sephadex G-50 of pronase-digested trypsinates from RSV-transformed and morphologically normal BHK_{21}/C_{13} cells grown in the presence of radioactive L-fucose. Cells were grown and harvested as stated in Materials and Methods. Appropriate trypsinates were pooled prior to pronase digestion. Pronase digestion was an described in Materials and Methods. Columns of Sephadex G-50 (0.8 × 100 cm) were equilibrated and developed with 0.1 M Tris-acetate (pH 9.0), 0.1% SDS, 0.1% mercaptoethanol, and 0.01% EDTA. Samples of 0.7 ml were collected at a flow rate of 13 ml/hr. BD represents fractions in which the Blue Dextran 2000 was eluted; ϕR represents fractions in which phenol red was eluted. ^3H cpm (●—●) and ^{14}C cpm (○—○). (a) Profiles obtained by cochromatographing pronase digests of pooled trypsinates from C_{13}/B_4 cells grown in the presence of 50 μCi of L-[^3H]fucose and BHK_{21}/C_{13} cells grown in the presence of 25 μCi of L-[^{14}C]fucose. (b) Profiles obtained by cochromatographing pronase digests of pooled trypsinates from C_{13}/SR_7 cells grown in the presence of 25 μCi of L-[^{14}C] fucose and BHK_{21}/C_{13} cells grown in the presence of 50 μCi of L-[^3H]fucose.

Dowex 50 (H^+) with water and passed over Dowex 1 (HCO_3^-). The recovery of ^{14}C radioactivity after this procedure was 93% while the recovery of ^3H radioactivity was 80%. The fractions were lyophilized and chromatographed with fucose as a carrier on cellulose plates (250 μ, Analtech, Wilmington, Del.). The plates were developed for 14 hr in both phases of ethyl acetate–pyridine–water (2:1:2, v/v) (Isherwood and Jermyn, 1951). Fucose was detected by spraying with aniline oxalate and the area of the cellulose was scraped from the plate into vials containing Aquasol (New England Nuclear, Boston) and counted in a liquid scintillation counter.

Source of Isotopes and Enzymes. L-[^{14}C]Fucose (50 mCi/mmole) was obtained from Calbiochem, Los Angeles, Calif. L-[^{14}C]Fucose (4.3 Ci/mmole), D-[^3H]glucosamine (1.3 Ci/mmole), L-[^3H]leucine (59.1 Ci/mmole), L-[^3H]valine (2.3 Ci/mmole), L-[^3H]phenylalanine (6.3 Ci/mmole, D-[^{14}C]glucosamine (10.7 mCi/mmole), L-[^{14}C]leucine (240 mCi/mmole), L-[^{14}C]valine (219 mCi/mmole), and L-[^{14}C]phenylalanine (376 mCi/mmole) were obtained from New England Nuclear (Boston, Mass.). Lyophilized three-times-crystallized trypsin (220–222 units/mg) and purified soybean trypsin inhibitor were obtained from Worthington Biochemicals (Freehold, N. J.). Pronase was obtained from Calbiochem. Hyaluronidase from bovine testes was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Results

Cochromatography on Sephadex G-200 of Trypsinates from Cells Grown in the Presence of Radioactive L-Fucose. The fucose-containing glycopeptides removed from the surface of BHK_{21}/C_{13} or C_{13}/B_4 cells by trypsinization were compared by cochromatography on Sephadex G-200. The resulting profile is seen in Figure 1. The radioactivity was eluted in three major regions. The most striking and consistent difference was found in the major peak which was eluted just after the Blue Dextran marker. The glycoproteins from the C_{13}/B_4 cells eluted in this region ahead of those from the BHK_{21}/C_{13} cells. The additional peak of radioactivity from BHK_{21}/C_{13} cells seen in the second major region of the profile was not always readily resolved. In all other aspects the profiles of radioactivity from the two cell lines were similar.

Cochromatography on Sephadex G-200 of Surface Membranes from Cells Grown in the Presence of Radioactive L-Fucose. Glycoproteins remaining on the surface membranes of cells following trypsinization were compared after solubilization of the isolated surface membranes as stated in Materials and Methods. Figure 2 shows the profiles obtained by cochromatographing the surface membranes isolated from BHK_{21}/C_{13} and C_{13}/B_4 cells grown in the presence of L-[^{14}C]-fucose or L-[^3H]fucose, respectively. The glycoproteins from both membrane fractions migrated as two major peaks. As seen when the trypsinates were compared, the glycoproteins from membranes of the virus-transformed C_{13}/B_4 cells were eluted ahead of those from the BHK_{21}/C_{13} cells.

Cochromatography on Sephadex G-50 of Pronase Digests of Trypsinates from Cells Grown in the Presence of Radioactive L-Fucose. Figure 3a shows the results obtained when the combined trypsinate from BHK_{21}/C_{13} cells grown in the presence of L-[^{14}C]fucose and C_{13}/B_4 cells grown in the presence of L-[^3H]fucose were digested with pronase and chromatographed on Sephadex G-50. The profile consists of two major regions, the first migrating just after the Blue Dextran is the area of major interest. Here it can be seen that the material from the

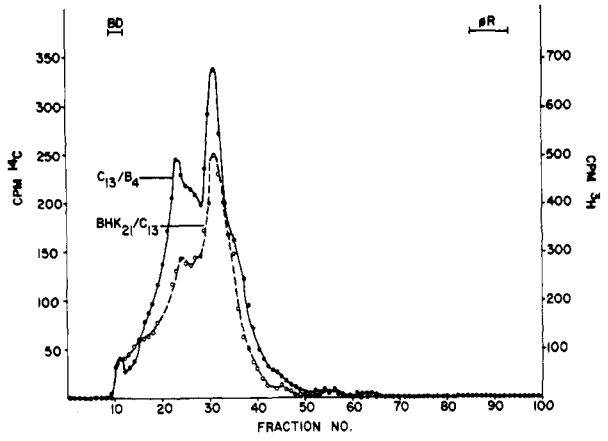

FIGURE 4: Cochromatography on Sephadex G-50 of pronase-digested purified surface membranes from C_{13}/B_4 and BHK_{21}/C_{13} cells grown in the presence of radioactive L-fucose. Membranes were prepared and digested with pronase (see Materials and Methods) from cells grown as described in Figure 2. Columns of Sephadex G-50 were prepared and developed as described in Figure 3. ^3H cpm from C_{13}/B_4 membranes (●—●) and ^{14}C cpm from BHK_{21}/C_{13} membranes (○---○). BD represents samples in which Blue Dextran 2000 was eluted; φR represents samples in which phenol red was eluted.

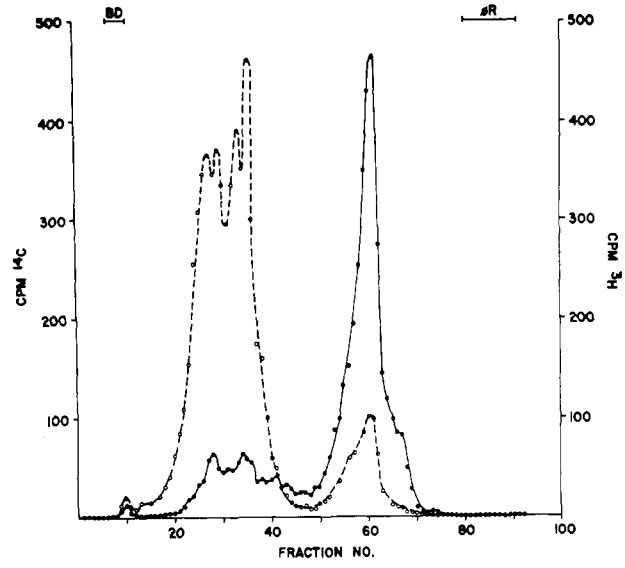

FIGURE 5: Cochromatography on Sephadex G-50 of pronase-digested washes from C_{13}/B_4 and trypsinates from C_{13}/B_4 cells grown in the presence of radioactive L-fucose. Cells were grown exactly as described in Figure 1 and Materials and Methods. Washes were prepared and pooled with trypsinates prior to digestion with pronase. Sephadex G-50 chromatography was as described in Figure 3. Wash, ^3H cpm (●—●), trypsinate ^{14}C cpm (○---○). BD, Blue Dextran; φR, phenol red.

C_{13}/B_4 cells has fucose-containing glycopeptides which were only present in limited amounts in the trypsinate from the morphologically normal BHK_{21}/C_{13} cells. Careful examination of the elution pattern of the control cells always revealed either a small peak or shoulder in this area. The irregularity of the first peak seen in this area is quite typical, suggesting that this peak contains several glycopeptides. The glycopeptides of apparently lower molecular weight which were eluted later from the column just ahead of the phenol red marker were present in both trypsinates. As will be seen in subsequent figures, these smaller glycopeptides could always be found in the trypsinates from fucose-labeled cells, but their relative quantity was variable. This material was completely dializable into H_2O during a 14-hr period.

Figure 3b shows that when a similar comparison was made between pronase-digested trypsinates from BHK_{21}/C_{13} cells and the same cells transformed by the Schmidt–Ruppin strain of RSV (C_{13}/SR_7), similar results are obtained. That is, the trypsinate from RSV-transformed cells grown in the presence of radioactive L-fucose contained more rapidly migrating glycopeptides which were found only in small quantities in the BHK_{21}/C_{13} cells. Cochromatography of trypsinates from C_{13}/B_4 and C_{13}/SR_7 cells showed that the pronase-digested glycopeptides from both lines of RSV-transformed cells migrated together on Sephadex G-50 columns.

Cochromatography on Sephadex G-50 of Pronase-Digested Surface Membranes from Cells Grown in the Presence of Radioactive L-*Fucose.* The elution profiles obtained by cochromatographing pronase digests of purified surface membranes from C_{13}/B_4 and BHK_{21}/C_{13} cells grown in the presence of radioactive L-fucose are shown in Figure 4. The surface membranes used in this experiment were prepared from cells of the same experiment from which the trypsinates shown in Figure 3a were obtained. Again, a relatively large peak containing radioactive fucose is seen in the transformed cell (fractions 15–28) and a much smaller peak is seen in the control in the same area.

A comparison of the profiles of the membranes and the trypsinate shows them to be similar. Only traces of the slower migrating glycopeptides (after fraction 50) were found in the membrane preparations following pronase digestion. More generally no radioactive material could be detected in this area.

Cochromatography on Sephadex G-50 of Pronase-Digested Washes from Cells Grown in the Presence of Radioactive L-*Fucose.* The washes from the L-fucose-labeled C_{13}/B_4 cells used in the experiments described in Figure 4 were pooled with the trypsinates of C_{13}/B_4 cells and digested with pronase. The profiles obtained following cochromatography on Sephadex G-50 are shown in Figure 5. The washes were apparently enriched in the slower migrating glycopeptides, and contained relatively little material migrating in the area of the higher molecular weight glycopeptides present in the membranes and trypsinates (Figures 3a and 4). The slower migrating material from the trypsinate was coeluted with that found in the wash. The two major fucose-containing peaks typical of trypsinates from RSV-transformed cells again were readily resolved. Washes from control and transformed cells gave essentially the same profile when cochromatographed on Sephadex G-50 following pronase digestion.

Cochromatography on Sephadex G-200 of Trypsinates from Cells Grown in the Presence of Radioactive D-*Glucosamine.* In order to determine if further differences existed in the glycoproteins from virus-transformed C_{13}/B_4 or C_{13}/SR_7 cells and BHK_{21}/C_{13} cells, the cells were grown in the presence of radioactive D-glucosamine. Figure 6a shows the profiles obtained when trypsinates from C_{13}/B_4 and BHK_{21}/C_{13} cells grown in the presence of D-[^3H]- and [^{14}C]glucosamine, respectively,

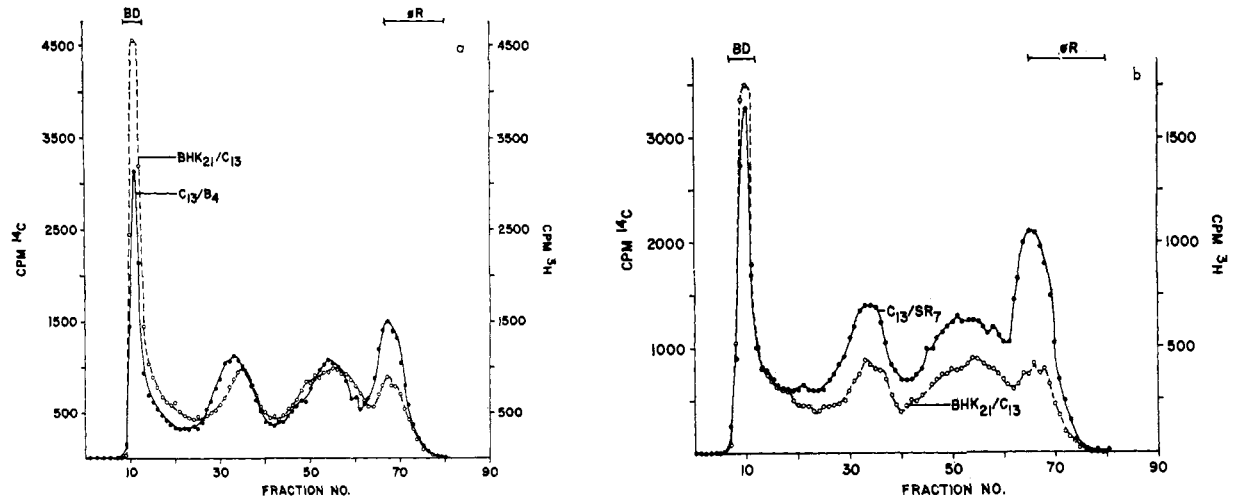

FIGURE 6: Cochromatography on Sephadex G-200 of trypsinates from RSV-transformed and morphologically normal BHK_{21}/C_{13} cells grown in the presence of radioactive D-glucosamine. Cells were grown on roller bottles as described in Materials and Methods in the presence of 50 μCi of D-[^3H]glucosamine (C_{13}/B_4; C_{13}/SR_7) or 25 μCi of D-[^{14}C]glucosamine (BHK_{21}/C_{13}]. Trypsinates were prepared and pooled prior to chromatography. The Sephadex G-200 column was prepared and developed as described in Figure 1. (a) Profiles obtained by cochromatographing trypsinates from D-[^3H]glucosamine-labeled C_{13}/B_4 cells (●—●), D-[^{14}C]glucosamine-labeled BHK_{21}/C_{13} cells (○—○). (b) Profiles obtained by cochromatographing trypsinates from D-[^3H]glucosamine-labeled C_{13}/SR_7 cells (●—●), and D-[^{14}C]glucosamine-labeled BHK_{21}/C_{13} cells (○—○). BD represents samples in which Blue Dextran 2000 was eluted; ϕR represents samples in which phenol red was eluted.

were cochromatographed on Sephadex G-200 columns. Four peaks of radioactivity were obtained. The first peak migrated with the Blue Dextran and probably consisted of mucopolysaccharides such as hyaluronic acid, as the amount of material migrating in this area was reduced by half following digestion with hyaluronidase. Mucopolysaccharides are known to be synthesized by fibroblasts in culture (Temin, 1968). In the second peak of Figure 6a it can be seen that the trypsinate from C_{13}/B_4 cells contained glucosamine-labeled material which migrated slightly ahead of material migrating in the same area from BHK_{21}/C_{13}. The patterns seen in the remainder of the profile were similar in the trypsinates from both cell lines.

When the trypsinates from C_{13}/SR_7 cells grown in the presence of D-[^3H]glucosamine were pooled and cochromatographed on Sephadex G-200 columns with the trypsinates from BHK_{21}/C_{13} cells grown in the presence of D-[^{14}C]glucosamine, the profiles shown in Figure 6b were obtained. There was no shift in any region of the profile comparable to that seen in the second peak in Figure 6a.

Cochromatography on Sephadex G-200 of Surface Membranes from Cells Grown in the Presence of Radioactive D-*Glucosamine.* The D-glucosamine-labeled material remaining on the surface membranes of C_{13}/B_4 and BHK_{21}/C_{13} cells following trypsin digestion was compared by cochromatography on Sephadex G-200. The resulting elution profiles are shown in Figure 7. The radioactivity from both membrane preparations was eluted in two major regions. Again, the material from the C_{13}/B_4 cells migrated ahead of material from BHK_{21}/C_{13} cells (fractions 20–35). The second major peak seen in the profile from C_{13}/B_4 membranes suggests an enrichment in material possibly present in reduced quantities in the BHK_{21}/C_{13} cells.

Cochromatography on Sephadex G-50 of Pronase Digests of Trypsinates from Cells Grown in the Presence of Radioactive D-*Glucosamine.* Figure 8a shows that after pronase digestion, material in the trypsinate from BHK_{21}/C_{13} and C_{13}/B_4 cells grown in the presence of radioactive D-glucosamine was eluted in three major regions. The first region contained material which was eluted with the Blue Dextran, and probably represents the mucopolysaccharides described in Figure 6a which also were eluted with the Blue Dextran. The material eluted in the middle region of the profile (fractions 10–20) showed again a difference between that obtained from the RSV-trans-

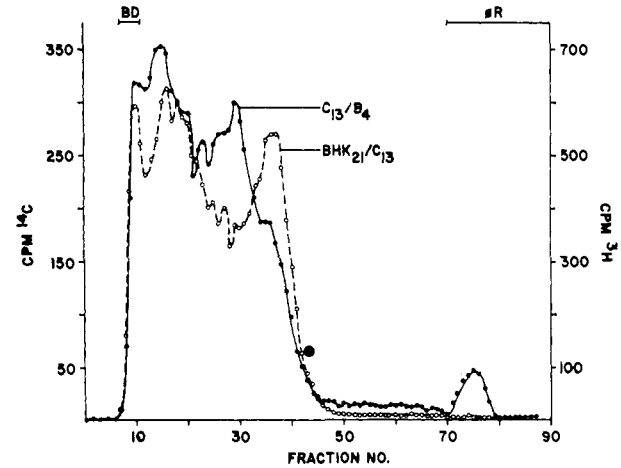

FIGURE 7: Cochromatography on Sephadex G-200 of purified surface membranes from C_{13}/B_4 and BHK_{21}/C_{13} cells grown in the presence of radioactive D-glucosamine. Cells were grown as described in Figure 6. Membranes were dissolved in 0.1% SDS and pooled prior to pronase digestion. Sephadex columns were prepared and developed as described in Figure 1. Profile from D-[^3H]-glucosamine-labeled C_{13}/B_4 cells (●—●); profile from D-[^{14}C]-glucosamine-labeled BHK_{21}/C_{13} cells (○---○). BD represents samples in which Blue Dextran 2000 was eluted; ϕR represents samples in which phenol red was eluted.

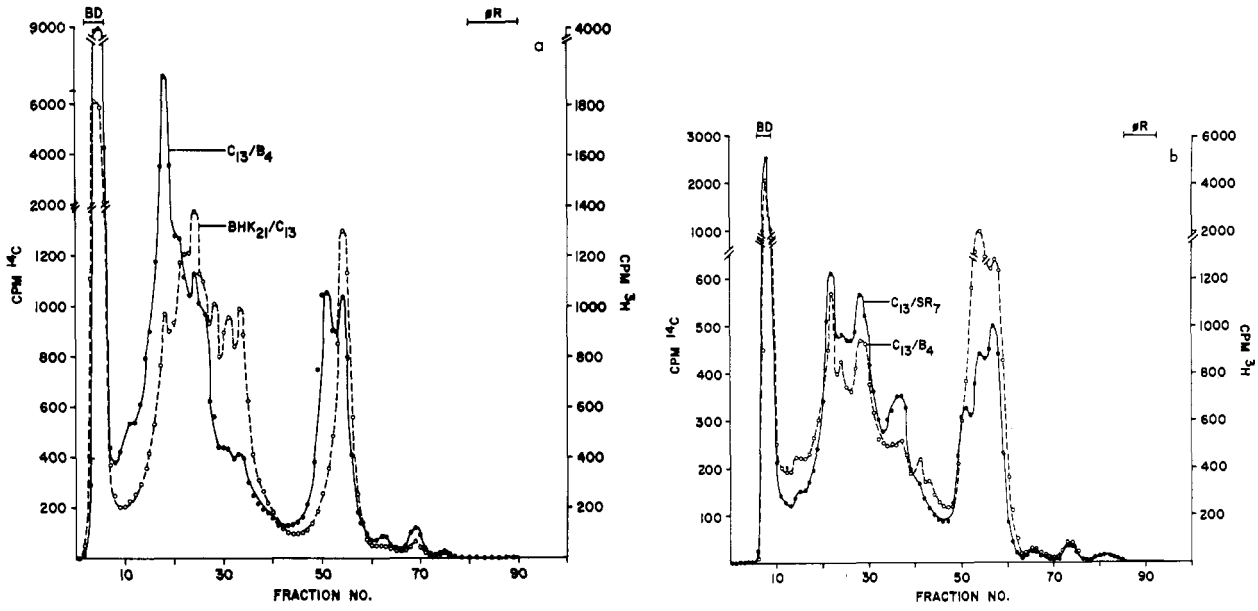

FIGURE 8: Cochromatography on Sephadex G-50 of pronase-digested trypsinates from RSV-transformed and morphologically normal BHK_{21}/C_{13} cells grown in the presence of radioactive D-glucosamine. Cells were grown as described in Figure 6. Trypsinates were prepared and pooled prior to pronase digestion. Sephadex G-50 columns were prepared and developed as described in Figure 3. (a) Profiles obtained upon cochromatography of pronase-digested pooled trypsinates from ^{3}H-labeled C_{13}/B_4 cells (●—●), and ^{14}C-labeled BHK_{21}/C_{13} cells (○—○). (b) Profiles obtained upon cochromatography of pronase-digested pooled trypsinates from ^{3}H-labeled C_{13}/SR_7 cells (●—●). and ^{14}C-labeled C_{13}/B_4 cells (○—○). Note scale changes. BD represents samples in which Blue Dextran 2000 was eluted; φR represents samples in which phenol red was eluted.

formed C_{13}/B_4 cells and that from the parental BHK_{21}/C_{13} cells. The material in this region from the C_{13}/B_4 cells was enriched in higher molecular weight material when compared to that from the BHK_{21}/C_{13} cells.

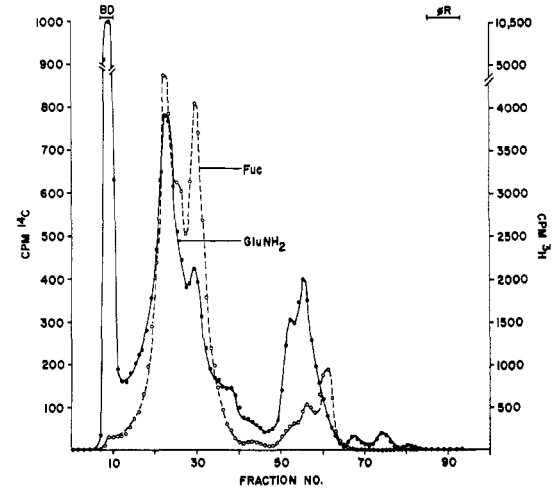

FIGURE 9: Cochromatography on Sephadex G-50 of a pronase digest of pooled trypsinates from C_{13}/B_4 cells grown in the presence of either radioactive L-fucose or D-glucosamine. C_{13}/B_4 cells were grown as stated in Materials and Methods in the presence of either 50 μCi of D-[^{3}H]glucosamine or 25 μCi of L-[^{14}C]fucose. Trypsinates were prepared, pooled, pronase digested, and chromatographed on Sephadex G-50 as described in Figure 3. Elution profiles of ^{3}H cpm (●—●); elution profile of ^{14}C cpm (○---○). BD represents samples in which Blue Dextran 2000 was eluted; φR represents samples in which phenol red was eluted.

Glycopeptides migrating just ahead of the phenol red marker (third group) were always present in D-glucosamine labeled trypsinates, but in variable amounts. The differences in the third group of glycopeptides derived from C_{13}/B_4 and BHK_{21}/C_{13} cells seen in Figure 8a were not always reproducible. In fact, details of the patterns of the glucosamine-labeled glycopeptides migrating in the various regions of the column, unlike those with labeled fucose, were difficult to reproduce from one experiment to another. Repeated chromatography of a single trypsinate, however, gave reproducible re-

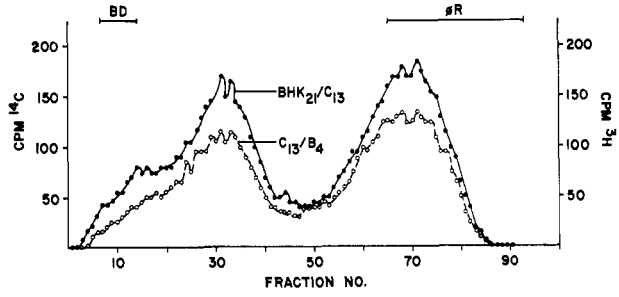

FIGURE 10: Cochromatography on Sephadex G-200 of trypsinates from C_{13}/B_4 and BHK_{21}/C_{13} cells grown in the presence of radioactive L-amino acids. Cells were grown as described in Materials and Methods in the presence of 100 μCi each of a mixture of L-[^{3}H]-valine, L-[^{3}H]leucine, and L-[^{3}H]phenylalanine (BHK_{21}/C_{13}) or 10 μCi each of L-[^{14}C]valine, L-[^{14}C]leucine, and L-[^{14}C]phenylalanine (C_{13}/B_4). Trypsinates were prepared, pooled, and cochromatographed on Sephadex G-200 columns as described in Figure 1. Profiles of ^{3}H cpm (●—●), and ^{14}C cpm (○---○). BD represents samples in which Blue Dextran 2000 was eluted; φR represents samples in which phenol red was eluted.

TABLE I: Amount of Radioactive L-Fucose Incorporated into Cells.[a]

Cell Line	Isotope	L-Fucose Incorpd (dpm/Cell)	Sp Act. (dpm/ μmole of Fucose)
	Experiment A		
BHK_{21}/C_{13}	^{14}C	0.0062	12.6×10^6
C_{13}/B_4	^{3}H	0.0140	26.9×10^6
	Experiment B		
BHK_{21}/C_{13}	^{3}H	0.0070	14.3×10^6
C_{13}/B_4	^{14}C	0.0053	10.2×10^6
C_{13}/SR_7	^{14}C	0.0040	14.8×10^6

[a] The amount of fucose per cell was determined by gas–liquid chromatography as described in Materials and Methods.

sults. We consistently found that the glycopeptides from the C_{13}/B_4 trypsinate which migrate in the second major region of the column always contained more of the larger glycopeptides migrating in the front of this region than did those from the BHK_{21}/C_{13} trypsinates.

A comparison of the pronase digests of trypsinates from C_{13}/B_4 and C_{13}/SR_7 cells grown in the presence of radioactive D-glucosamine is shown in Figure 8b. The resulting profiles were very similar, suggesting that the material from C_{13}/SR_7 cells was enriched in the more rapidly migrating glycopeptides.

Cochromatographing pronase digests of trypsinates from C_{13}/B_4 cells grown in the presence of D-[^{3}H]glucosamine with that of the same cells grown in the presence of L-[^{14}C]fucose resulted in the profile shown in Figure 9. Here it is seen that the more rapidly migrating D-glucosamine-labeled glycopeptides were eluted with the L-fucose-containing material found in increased quantities in the transformed cells (Figure 3).

Cochromatography on Sephadex of Material from Cells Grown in the Presence of Radioactive Amino Acids. Cells were grown in the presence of a mixture of either L-[^{14}C]valine, L-[^{14}C]leucine and L-[^{14}C]phenylalanine or L-[^{3}H]valine, L-[^{3}H]-leucine and L-[^{3}H]phenylalanine. The elution profile obtained when trypsinates from BHK_{21}/C_{13} and C_{13}/B_4 cells were co-chromatographed on Sephadex G-200 is shown in Figure 10.

TABLE II: Percentage of Radioactive L-Fucose found in Various Fractions Following Trypsin Digestion.[a]

Cell Line	% of Total dpm in Membranes	Trypsinate	Wash	Remainder
BHK_{21}/C_{13}	18	22	2	58
C_{13}/B_4	20	15	2	63
C_{13}/SR_7	10	20	3	69

[a] The percentage of radioactive L-fucose found in each fraction was determined from the cells used in Table I.

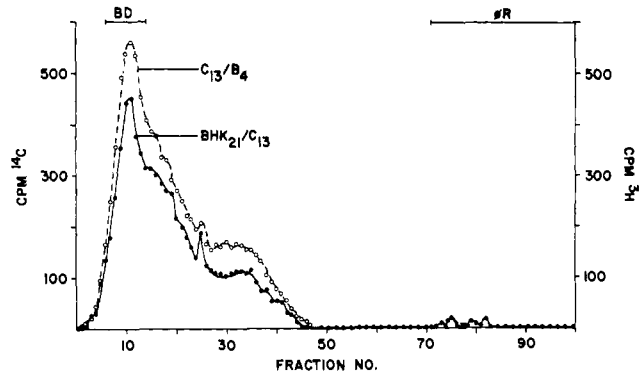

FIGURE 11: Cochromatography on Sephadex G-200 of purified surface membranes from C_{13}/B_4 and BHK_{21}/C_{13} cells grown in the presence of radioactive L-amino acids. Membranes were isolated from the cells used in the experiment described in Figure 10. Membranes were dissolved in 0.1% SDS and pooled prior to cochromatography. Columns were prepared and developed as described in Figure 1. ^{3}H cpm from BHK_{21}/C_{13} membranes (●—●), ^{14}C cpm from C_{13}/B_4 membranes (○---○). BD represents samples in which Blue Dextran 2000 was eluted; φR represents samples in which phenol red was eluted.

The two profiles were very similar indicating no major difference in the polypeptides removed from these cells with trypsin. When the surface membranes from these same cells were solubilized in SDS and cochromatographed on Sephadex G-200 the profiles shown in Figure 11 were obtained. Again, no major differences were seen.

To examine the extent of the pronase digestion, trypsinates or surface membranes from BHK_{21}/C_{13} and C_{13}/B_4 cells grown in the presence of radioactive leucine, valine, and phenylalanine were digested with pronase and the resulting polypeptides cochromatographed on Sephadex G-50. These results (Figure 12) indicate no preferential digestion of trypsinates from either cell line. No radioactivity was found in the region of the elution pattern where the major fucose or glucosamine-containing glycopeptides were found (fractions 15–50). Most

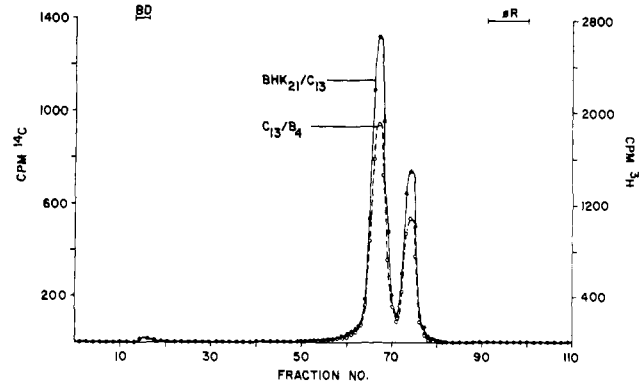

FIGURE 12: Cochromatography on Sephadex G-50 of pronase-digested trypsinates from cells frown in the presence of radioactive L-amino acids. Pooled trypsinates described in Figure 10 were digested with pronase and cochromatographed on Sephadex G-50. Profiles obtained from ^{3}H-labeled trypsinates from BHK_{21}/C_{13} cells (●—●), and ^{14}C-labeled trypsinates from C_{13}/B_4 cells (○—○). BD represents samples in which Blue Dextran 2000 was eluted; φR represents samples in which phenol red was eluted.

TABLE III: Sialic Acid Removed from Cells During Trypsin Digestion.[a]

Cell Line	Sialic Acid: Cells (μmoles $\times$ 10^{-10}/Cell)	Sialic Acid: Membrane (μmoles $\times$ 10^{-10}/Membrane)	% of Total Sialic Acid: Trypsinate	% of Total Sialic Acid: Wash
BHK_{21}/C_{13}	15.0	3.7	23.8	2.1
C_{13}/B_4	35.1	3.3	22.9	3.0
C_{13}/SR_7	15.3	3.9	20.4	3.1

[a] Cells were grown as stated in Materials and Methods. Sialic acid determinations were made on the various fractions (whole cells, trypsinate, and wash) following trypsinization as described in Materials and Methods. The sialic acid per cell was determined after adding the total sialic acid content of whole cells, trypsinate, and wash. Sialic acid content of membranes was determined using surface membranes prepared by the zinc ion method.

of the radioactivity was eluted as two peaks just ahead of the phenol red marker. Similar results were obtained when the purified membranes from these same cells were digested with pronase and cochromatographed.

Comparisons of the Amount of Radioactive Fucose Incorporated into BHK_{21}/C_{13}, C_{13}/B_4, and C_{13}/SR_7 Cells. Examination by thin-layer chromatography of pronase-digested trypsinates from cells grown in the presence of L-[^{14}C]- or L-[^{3}H]-fucose showed that 90% of the ^{14}C and 85% of the ^{3}H was recovered in the fucose area of the chromatogram. Less than 5% of either isotope was recovered in other areas of the chromatogram.

In order to compare the amount of L-[^{14}C]- or L-[^{3}H]fucose incorporated into the various cells, the specific activity of fucose in the cells was determined. Results from two experiments in which the isotopes were interchanged are shown in Table I. It was found that the specific activity of the fucose in the three cell lines was rather similar. The slight variability of the specific activity noted with the C_{13}/B_4 cells did not appear to be reflected in altered chromatographic profiles. The profiles obtained by chromatographing the various fractions were the same whether the cells were grown in the presence of L-[^{14}C]- or L-[^{3}H]fucose.

Comparison of the Extent of Trypsinization of the Various Cell Lines. Table II shows the percentage of the total radioactive fucose removed from the surface of the cell lines during trypsin digestion (trypsinates and washes) as well as that remaining associated with the surface membranes. The per cent of radioactive L-fucose removed by trypsin from the BHK_{21}/C_{13} and C_{13}/SR_7 cells was consistently higher than the amount removed from the C_{13}/B_4 cells. The washes from all cell lines contained about the same per cent of radioactivity. In order to get some idea of the extent of removal of glycopeptides in general by trypsin digestion, the amount of sialic acid released into the trypsinate was measured. Table III shows that in all cases, a similar percentage of sialic acid was released following trypsin digestion, and that similar amounts of sialic acid remained on the purified surface membranes from the cells digested with trypsin.

Discussion

Evidence is accumulating which indicates that the glycoproteins found on the surface membranes of cells are associated with a variety of important biological phenomena. It has been known for some time that they are the determining factors in the blood group antigens (Watkins, 1966; Lloyd and Kabat, 1968). More recently they have been implicated as important to histocompatability antigens (Muramatsu and Nathenson, 1970). Their participation in cellular regulatory phenomena is implied by the fact that glycoproteins are the receptors for the mitogenic interaction of phytohemagglutinin with lymphocytes (Kornfeld and Kornfeld, 1969). The removal of carbohydrates from cell surfaces by neuraminidase or other glycosidases results in alterations of cellular interactions (Gesner and Ginsburg, 1964; Woodruff and Gesner, 1967; Kemp, 1968).

These observations have stimulated comparative investigations of the glycolipids and glycoproteins of viral-transformed and normal cells. Alterations in the total glycolipid content of cells has been reported by Hakomori *et al.* (1968) and Mora *et al.* (1969). Although the reports are somewhat contradictory they both demonstrate changes in the glycolipid content of cells upon transformation. Wu *et al.* (1969) have found a decrease in the neutral and amino sugars of spontaneously transformed and SV40-transformed 3T3 cells when compared to normal 3T3 cells. Meezan *et al.* (1969) showed that the glycopeptides obtained by pronase digestion of various subcellular fractions from control SV_{40}-transformed 3T3 cells grown in the presence of radioactive D-glucosamine could be distinguished by gel filtration. This suggested that the glycopeptides from transformed and control cells were different. The results reported here extend these findings to the surface membrane of cells transformed by RNA-containing tumor viruses. In both cases, increased quantities of more rapidly migrating glycopeptides were found in preparations from transformed cells.

We have compared the glycoproteins from the surface of a continuous hamster cell line, BHK_{21}/C_{13} with those of BHK_{21}/C_{13} cells transformed with two different strains of RSV. These cells have certain empirical cultural characteristics which strongly suggest that transformation of BHK_{21}/C_{13} cells with RSV results in the alteration of components of the surface membrane. The morphologically normal parental BHK_{21}/C_{13} cells grow as typical fibroblasts, sticking well to glass, failing to form colonies in soft agar, and forming a single sheet of cells upon growth to confluency. The C_{13}/B_4 cells, on the other hand, are large, rounded cells which stick less well to glass, readily

form colonies in soft agar and grow in stacks upon one another as the cultures become crowded. These morphological differences should then be reflected by chemical differences.

Cochromatography of the trypsinates from control and RSV-transformed cells following digestion with pronase revealed that the material from the transformed cells was enriched with fucose-containing glycopeptides which were eluted ahead of the major fucose-containing glycopeptides from the control cells. Trypsinates from control cells also contained material which migrated ahead of the major fucose-containing glycopeptides, but never as much as the transformed cells. As yet there is no evidence that the glycopeptides from the two cell types are identical even though they behave the same on Sephadex G-50. Trypsinates from transformed cells grown in the presence of radioactive D-glucosamine also showed an enrichment of higher molecular weight glycopeptides which coeluted from Sephadex G-50 with the fucose-containing glycopeptides.

It should be pointed out that the pronase digestion of trypsinates from C_{13}/B_4 cells seemed to contain more of the higher molecular weight glycopeptides than similar preparations from C_{13}/SR_7 cells. The latter containing only slightly more than was found in control cells (see Figure 3b). The material from C_{13}/SR_7 cells, unlike that from the control cells, was always readily resolved from the remaining glycopeptides. This suggests an enrichment in glycopeptides not found, or present in reduced quantities in control cells. This suggestion is strengthened when the profiles of material obtained from cells grown in the presence of radioactive glucosamine shown in Figure 8 are compared. The material from C_{13}/SR_7 cells shown in Figure 8b was clearly more enriched in the higher molecular weight glycopeptides than similar material from control cells shown in Figure 8a.

The pattern seen using radioactive fucose was more simple and clear than that obtained after labeling with radioactive D-glucosamine. This may be due to the fact that glucosamine may serve as a precursor to carbohydrates such as sialic acid, *N*-acetylglucosamine, and *N*-acetylgalactosamine, while exogenous fucose is found in the cell as such.

We have observed that the amount of these glycopeptides synthesized in both the control and RSV-transformed cells fluctuated with the state of growth of the cells (unpublished observations). The amount of the higher molecular weight material decreased as the monolayers became heavy, *i.e.*, when the number of cells per bottle reached 3×10^8. For the experiments reported here, the cell count was much lower at the time of harvesting, *i.e.*, 0.9×10^8–1.2×10^8 cells/bottle. This would suggest that these glycopeptides might be involved in surface changes which occur as cells proceed through mitosis or come in contact with one another. However, trypsinates from transformed cells, regardless of the state of growth during which radioactive L-fucose was present in the culture, were always enriched in the more rapidly migrating glycopeptides relative to the control BHK_{21}/C_{13} cells. This has been the case in every one of the 16 different trypsinates examined in the course of this and other studies.

When trypsinates from control and transformed cells grown in the presence of radioactive amino acids were cochromatographed on Sephadex G-200, no major differences were observed. This was in contrast to the results obtained from similar comparisons of trypsinates and surface membranes from cells grown in the presence of radioactive L-fucose. It cannot be concluded, however, that the polypeptides released from control and transformed cells by digestion with trypsin are identical. This problem will have to be resolved using methods of polypeptide analysis more sensitive than chromatography on Sephadex G-200.

The following observations suggest that the difference reported here between the glycopeptides from the surface of morphologically normal and RSV-transformed hamster cells reflect actual difference in the surface material from these cell lines. First, the percentage of the total material containing sialic acid released from the cell surface by trypsinization was the same for all three cell lines. Second, the specific activity of L-fucose in all three cell lines was about the same. Third, reversing the isotope, that is growing transformed cells in the presence of L-[^{14}C]fucose rather than L-[^{3}H]fucose, did not alter the chromatographic profiles. Fourth, trypsinization resulted in the release of slightly more radioactive fucose-containing glycopeptides from the BHK_{21}/C_{13} cells than from the C_{13}/B_4 cells. This, and the decreased amounts of the higher molecular weight glycopeptides in purified BHK_{21}/C_{13} membranes would suggest that the relatively smaller amounts of these glycopeptides in BHK_{21}/C_{13} trypsinates was a reflection of the lower levels of this material in the cell surface structure and not the result of incomplete trypsinization of superficial layers. Fifth, digestion of surface material from the parental and RSV-transformed cells with pronase was complete as measured by the absence of radioactive polypeptides eluting in the same region from Sephadex G-50 columns as the major fucose-containing glycopeptides. The smaller polypeptides attached to the oligosaccharides probably lacked leucine, phenylalanine, or valine and therefore went undetected in our system.

These results suggest the presence of additional or altered glycoproteins on the surface of transformed cells. These alterations might conceivably be related to surface phenomenon such as loss of contact inhibition, agglutinability by wheat germ agglutinin (Burger, 1969), concanavaline A (Inbar and Sachs, 1969), and the presence of new transplantation antigens (Klein, 1968). They could also reflect the production of virus specific glycopeptides by transformed cells. This possibility is weakened by the fact that we have observed similar alterations in the glycopeptide patterns from BHK_{21}/C_{13} cells transformed by polyoma virus (unpublished observations).

We are extending these observations to other cells transformed by RNA and DNA tumor viruses and are beginning detailed chemical and immunological analyses of the fucose-containing glycoproteins from these cells.

Acknowledgments

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Ester and Ether-Linked Lipids in the Mandibular Canal of a Porpoise (*Phocoena phocoena*). Occurrence of Isovaleric Acid in Glycerolipids*

Usha Varanasi and Donald C. Malins

ABSTRACT: High proportions of isovaleric acid (40.5 mole %) and long-chain iso acids, such as isopentadecanoic acid (4.8 mole %), are present in the neutral glycerolipids of the mandibular canal of the porpoise (*Phocoena phocoena*). Although isovaleric acid, a product of leucine metabolism, is readily esterified in triglyceride biosynthesis the isopentyloxy structure was not detected in the alkyl chains of glyceryl ethers or the dialkoxypentane fraction of the diol lipids. These findings suggest that isovaleric acid, unlike longer chain structures, is not readily reduced and incorporated into alkyl moieties. The apparent absence in the mandibular canal of C_{20} and C_{22} unsaturated acids characteristic of marine organisms suggests that lipid biosynthesis is not significantly dependent on dietary polyenoic acids.

The mandible of the porpoise (*Phocoena phocoena*) contains a cavity filled with fatty tissue. This tissue plays an important yet undefined role in the echolocation system of this cetacean (Norris, 1964). The presence of such a deposit in the mandible may imply a biochemical function for lipids in sound transmission that is reminiscent of the role for these compounds in the photochemistry of sight (Adams, 1969; Erhardt *et al.*, 1966). Nevertheless, very little is known about the composition of the mandible tissue. Early workers, however, have noted large proportions of isovaleric acid in the total lipids of some cetaceans (Hilditch and Williams, 1964).

Two recent papers from this laboratory have revealed that the mandible tissue contains unique diol lipids (dialkoxyalkanes) (Varanasi and Malins, 1969) and unusual wax

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