Comparative Studies on the Carbohydrate-Containing Membrane Components of Normal and Virus-Transformed Mouse Fibroblasts. II. Separation of Glycoproteins and Glycopeptides by Sephadex Chromatography*

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ABSTRACT: Analysis of membrane carbohydrates has revealed that following SV-40 virus transformation of mouse fibroblast 3T3, there is a marked decrease in most neutral and amino sugars, especially sialic acid and *N*-acetylgalactosamine. To examine this change in the composition of membrane glycoproteins in greater detail, we have fractionated the labeled membrane fractions derived from 3T3 and transformed 3T3 lines on Sephadex G-150 in the presence of sodium dodecyl sulfate. In addition, membrane glycoproteins were digested with the proteolytic enzyme, Pronase, followed by the fractionation of the glycopeptide mixture on Sephadex G-50. The possible mechanisms by which these changes in the composition of membrane glycoproteins are brought about are discussed.

In the preceding paper (Wu *et al.*, 1969) we described a double-labeling technique for analyzing the membrane glycoproteins of cultured animal cells. These studies showed that following tumor virus transformation there is a marked change in the glycoprotein composition of 3T3 cells with a decrease in most neutral and amino sugars, expecially in sialic acid and *N*-acetylgalactosamine. These results are in agreement with analyses carried out in several laboratories which have shown a decrease in sialic acid content in tumor virus transformed cells as compared with the cell lines from which they were derived (Ohta *et al.*, 1968). In a detailed study, Hakamori and Murakami (1968) showed that the major glycolipid of BHK 21-C13 fibroblasts was hematoside (*N*-acetylneuraminyllactosylceramide) while the main glycolipid of cells derived by polyoma virus transformation of this cell was lactosylceramide. The amount of ceramide is comparable in the two cell lines and the structures are the same except for the much smaller amount of *N*-acetylneuraminic acid attached to the lipid from the transformed line. Since *N*-acetylneuraminic acid is always present in nonreducing terminal positions, Hakamori and Murakami suggest that carbohydrate chains in materials from tumor cells may often be "incomplete." As further evidence for this, they cite work with adenocarcinomas which shows that loss of blood group A and B activities is associated with the appearance of H and Le^a glycolipids. These changes would correspond to the loss of *N*-acetylgalactosaminyl, galactosyl, and $\alpha(1\rightarrow2)$-L-fucosyl residues from the glycolipids and/or glycoproteins of the carcinoma.

Our finding of lower *N*-acetylneuraminic acid, *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose, and mannose per milligram of protein in membranes of transformed cells would be compatible with the idea that complex carbohydrate chains are left uncompleted in transformed cells. However, other explanations for this change in composition should be considered including the following as the simplest possibilities. (1) Following transformation all membrane glycoproteins are formed in exactly the same proportion as before transformation but are formed in smaller total amount. This possibility can be ruled out by the differences in the ratios of various sugars found before and after transformation (Wu *et al.*, 1969). (2) Exactly the same glycoproteins are formed after transformation but in different proportions. If each glycoprotein carried different oligosaccharides, some containing more sugars than others, then it is obvious that a decrease in a carbohydrate-rich glycoprotein could lead to a depletion in carbohydrate composition even though the total amount of protein in the membrane remained constant. (3) There are actually different glycoproteins in the transformed 3T3 cells which were not present in 3T3 cells prior to transformation. (4) The glycoproteins may differ structurally and in their relative amounts.

Since answers to these questions might give insight into the changes in the cell surface membrane that are brought about by transformation, we have fractionated the labeled membrane fractions derived from 3T3 and transformed 3T3 lines on Sephadex G-150 in the presence of sodium dodecyl sulfate. Experiments by Kennedy and coworkers (T. Jones, personal communication) have shown that this procedure separates membrane proteins largely on the basis of molecular weight. An alternative method of fractionation was digestion with the proteolytic enzyme Pronase followed by separation of glycopeptides on Sephadex G-50.

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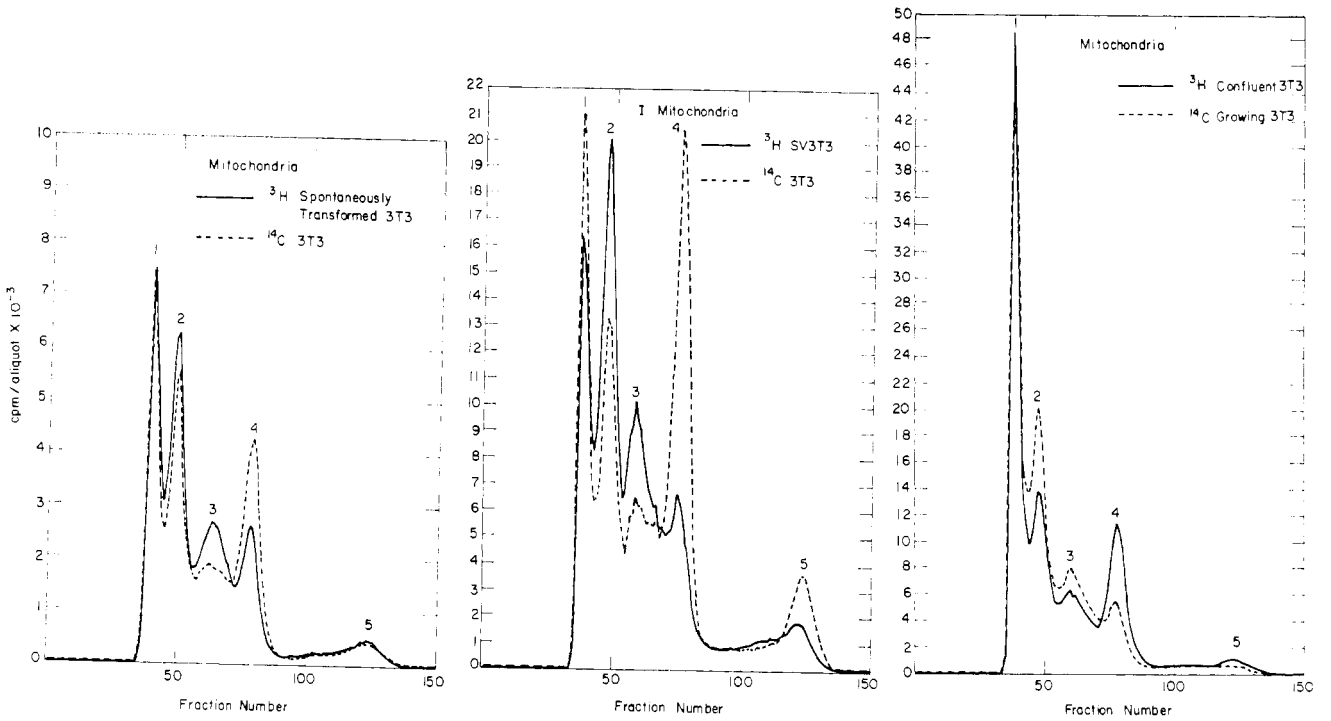

FIGURE 1: Fractionation of glycoproteins on Sephadex G-150. Mitochondrial suspensions doubly labeled with [^{3}H]- and [^{14}C]glucosamine *in vivo* were solubilized with 2% sodium dodecyl sulfate in 0.025 M Tris buffer (pH 7.4) containing 7 mM 2-mercaptoethanol. After centrifugation at 104,000*g* for 1 hr at 2°, the supernatant was applied to a Sephadex G-150 column (2.7 × 82 cm) which had been equilibrated with 0.01 M sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 1% sodium dodecyl sulfate. The column was eluted with the same buffer at a flow rate of 2 ml/hr. Fractions of 3 ml were collected and aliquots of 1–2 ml were counted. The curves were plotted after normalizing the ^{3}H/^{14}C ratio of the unfractionated sample to unity so that the total counts per minute of ^{3}H and ^{14}C isotopes are equal. (—) ^{3}H cpm and (– – –) ^{14}C cpm. Left: mitochondrial fractions of [^{3}H]glucosamine-labeled ST-3T3 and [^{14}C]glucosamine-labeled 3T3; center: ^{3}H-labeled SV-40-3T3 and ^{14}C-labeled 3T3; right: ^{3}H-labeled confluent 3T3 and ^{14}C-labeled growing 3T3.

Experimental Procedures

Pronase (*Streptomyces griseus* protease, 45,000 proteolytic units/g) was obtained from the California Biochemical Corp. Sephadex G-50 (fine) and G-150 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

The history of mouse fibroblast lines used in the present study, the culture of these cells, the labeling of cells with radioactive glucosamine and the subsequent subcellular fractionation were described in detail in the preceding paper (Wu *et al.*, 1969). For the labeling of 3T3 and SV-40-3T3 with radioactive glucosamine, these two cell lines were grown and labeled separately with [6-^{3}H]glucosamine and [1-^{14}C]glusosamine for 5 days. After the cells were harvested and washed, they were mixed to form two groups, mixtures I and II, each containing approximately equal number of counts per minute of ^{3}H and ^{14}C. Mixture I was composed of ^{3}H-labeled SV-40-3T3 and ^{14}C-labeled 3T3 whereas mixture II represented the criss-cross control, ^{3}H-labeled 3T3 and ^{14}C-labeled SV-40-3T3.

Sephadex G-150 Column Chromatography of Membrane Glycoproteins. Dry Sephadex G-150 was suspended in 0.01 M sodium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 1% sodium dodecyl sulfate, and allowed to swell at 60° for 5 hr followed by stirring at room temperature for 3 days. A column of 2.7 × 82 cm was packed with a pressure head of 15 cm. Glucosamine-labeled membrane fractions were extracted with 2% sodium dodecyl sulfate in 0.025 M Tris buffer (pH 7.4) and 7 mM mercaptoethanol for 30 min at 37°, followed by centrifugation at 104,000*g* for 60 min. The supernatant fluid (2 ml) was introduced onto the top of the column and the elution was carried out with the same buffer (phosphate–EDTA–sodium dodecyl sulfate–mercaptoethanol) at a flow rate of 2 ml/hr. Fractions (3 ml) were collected and aliquots of 1–2 ml were counted with 10 ml of Patterson–Greene scintillation fluid for the determination of the ^{3}H/^{14}C ratio of each fraction (Patterson and Greene, 1965).

Pronase Digestions of Subcellular Fractions and Sephadex G-50 Fractionation (Spiro, 1965). The subcellular fractions were dialyzed and adjusted to a volume of 10–12.5 ml in pH 7.8 sodium phosphate or Tris buffer (0.2 M). The digests were also made 0.0015 M in $CaCl_2$ and 0.4 ml of a 20-mg/ml solution of Pronase was added to start the digestion. A few drops of toluene were added to prevent bacterial growth. The digestions were carried out for about 120 hr at 37°. Additional amounts of Pronase were added at 48 and 72 hr after the start of the digestion (4 mg each time). At the completion of the digestions, insoluble material was removed by centrifugation and the supernatant fluids were concentrated to 3 ml for chromatography on Sephadex G-50.

Chromatography of the Pronase-digested subcellular fractions was carried out on a 2.5 × 66 cm Sephadex G-50 fine column which was eluted with 0.05 M ammonium acetate. The void volume and the total volume of the column were determined by running through Blue Dextran and sodium chloride. Fractions of 3 ml were collected every 20 min. Isolation of

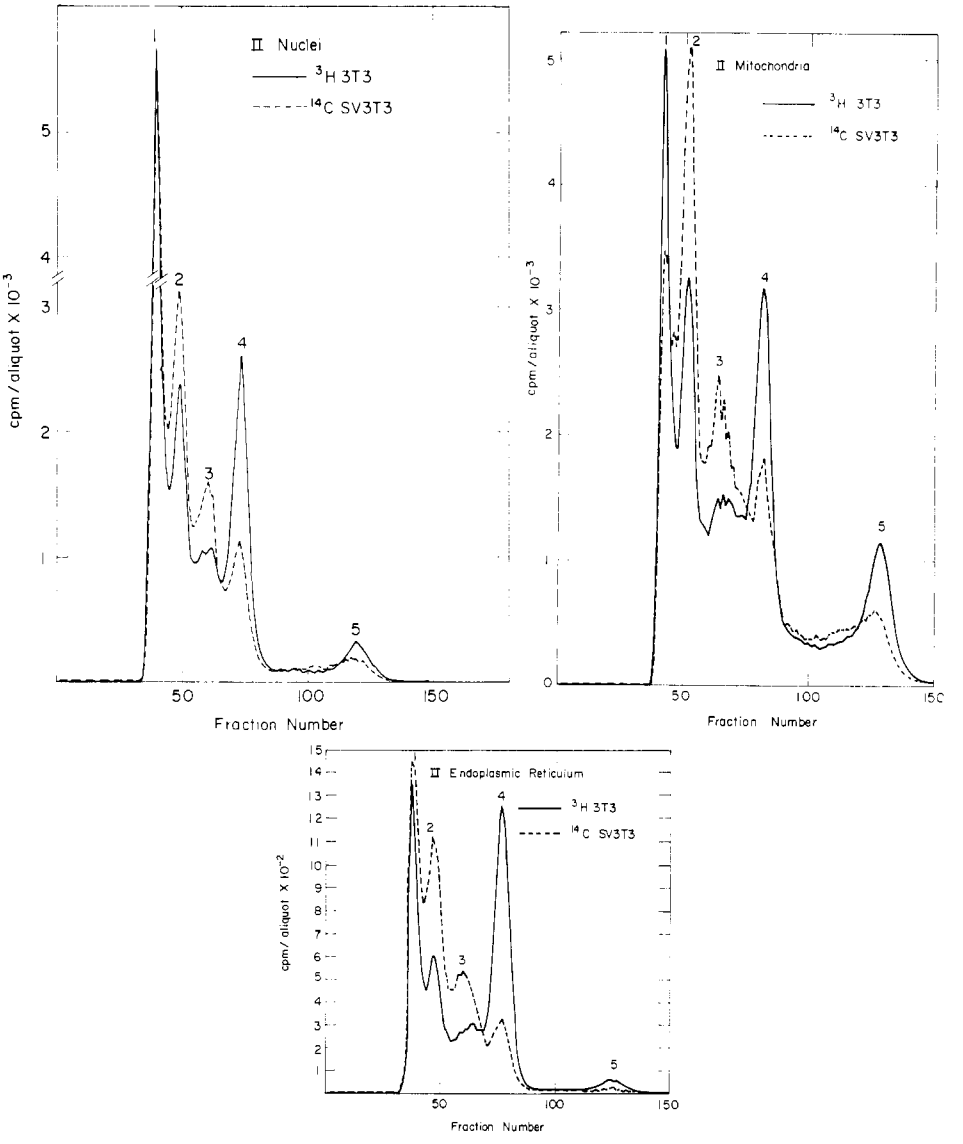

FIGURE 2: Fractionation of membrane glycoproteins on Sephadex G-150. 3T3 and SV-40-3T3 were labeled with [^{3}H]glucosamine and [^{14}C]-glucosamine, respectively. Cells were harvested and fractionated as described previously. Membrane glycoproteins were solubilized with 2% sodium dodecyl sulfate and fractionated on a Sephadex G-150 column in the presence of 1% sodium dodecyl sulfate. Details of the experiments were identical with those described in the legend to Figure 1. (—) ^{3}H cpm and (- - -) ^{14}C cpm. Upper left: glycoprotein from nuclei; upper right: glycoproteins from mitochondria; lower: glycoproteins from endoplasmic reticulum.

sialic acid and hexosamine from acid hydrolysates of glycopeptide fractions and measurements of ^{3}H/^{14}C ratios were the same as described in the preceding paper (Wu *et al.*, 1969).

Results

Fractionation of Membrane-Bound Glycoproteins by Sephadex G-150 Chromatography in the Presence of Sodium Dodecyl Sulfate. Sephadex column chromatography in sodium docecyl sulfate containing buffers has proved to be useful in fractionating membrane-bound proteins according to size (T. Jones, personal communication). Mitochondrial fractions doubly labeled with glucosamine were solubilized with 2% sodium dodecyl sulfate; the extracted material was subsequently fractionated on a Sephadex G-150 column with phosphate buffer containing 1% sodium dodecyl sulfate. The profiles of radioactive glycoproteins in mitochondria from the following mixtures are shown in Figure 1: ^{3}H-SV-40-3T3 and ^{14}C-3T3, ^{3}H-ST-3T3 and ^{14}C-3T3, and ^{3}H-confluent 3T3 and ^{14}C-growing 3T3. These curves were plotted after normalizing the ^{3}H/^{14}C ratio of the unfractionated sample to unity. It is clear that the glycoprotein profile in mitochondria of SV-40-3T3 is significantly different from that of 3T3 mitochondria. Four peaks were noted. Peak 1 represented glycoproteins totally excluded by Sephadex G-150. Peaks 2 and 3 were found to be present in larger amounts in SV40-3T3 and ST-3T3 than in 3T3 whereas the reverse was true for peaks 4 and 5. Peak 4, of approximate molecular wieght of 20,000 is of particular interest, not only because it was found in much larger quantities in 3T3 than transformed 3T3, but also due to the fact that it was present in even larger amount when 3T3 was labeled for five days after it had approached confluent growth. The nature

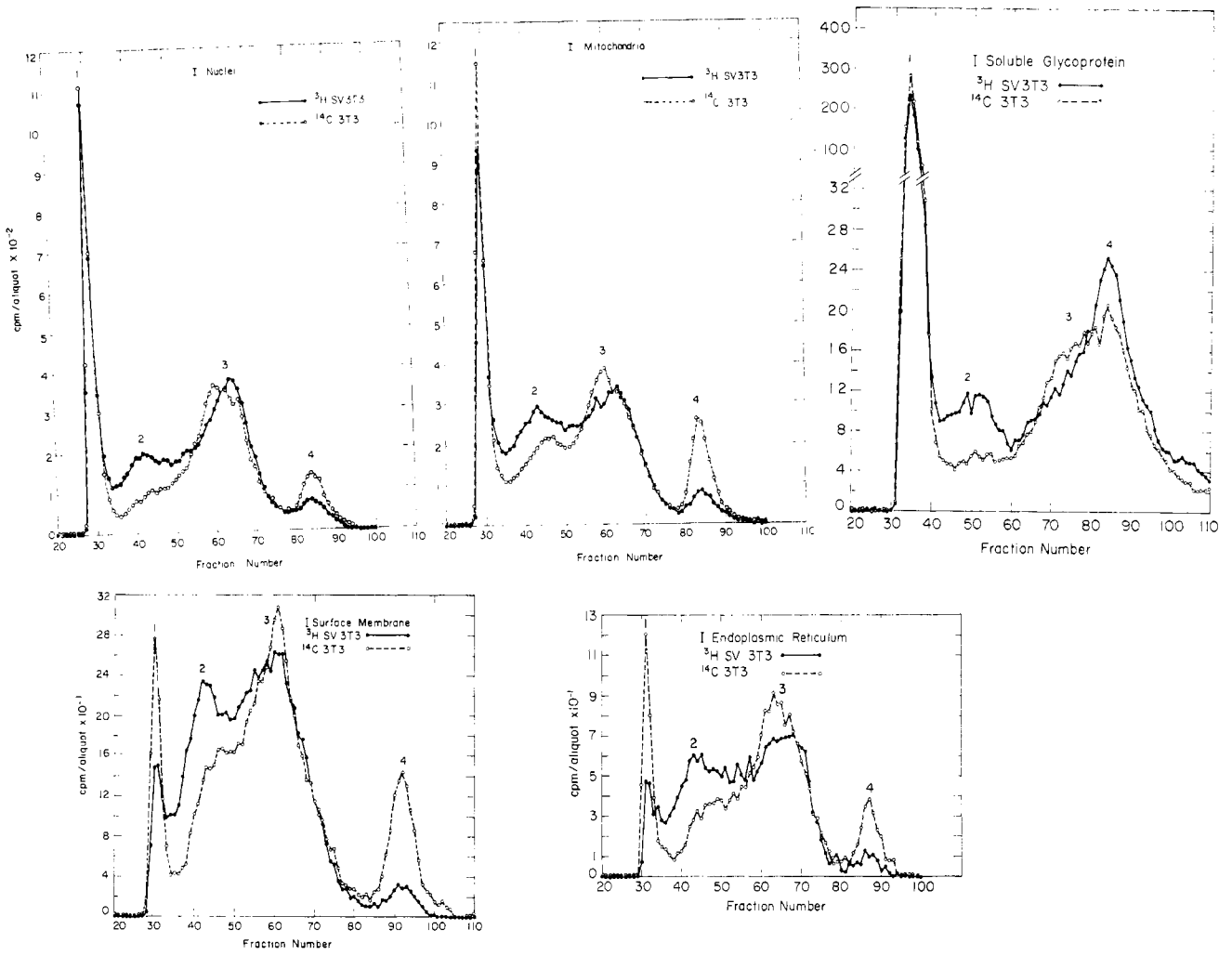

FIGURE 3: Fractionation of glycopeptides on Sephadex G-50. [^{3}H]Glucosamine-labeled SV-40-3T3 and [^{14}C]glucosamine-labeled 3T3 cells were mixed and fractionated into nuclei, mitochondria, surface membrane, endoplasmic reticulum, and soluble fractions as described previously. Each fraction containing doubly labeled glycoproteins was digested with Pronase and the resulting glycopeptide mixture was fractionated on a Sephadex G-50 column (2.5 × 66 cm). The column was eluted with 0.05 M ammonium acetate. Fractions of 3 ml were collected every 20 min and an aliquot of each fraction was taken for the measurements of ^{3}H and ^{14}C. The curves have again been normalized to a ^{3}H/^{14}C ratio of 1. (●—●) ^{3}H cpm and (○- - -○) ^{14}C cpm.

of this glycoprotein species (or mixture of glycoproteins) and the physiological variation of the amount of this species with respect to both transformation and status of contact inhibition are presently under investigation.

As can be seen in Figure 2, the difference in the Sephadex G-150 profiles of membrane-bound glycoproteins from 3T3 and SV-40-3T3 was seen in all particulate fractions. In all fractions, the differences in peak four were most prominent. It is also clear from Figures 1 and 2 that these differences in the profiles on Sephadex G-150 columns were remarkably similar when the labeling pattern was reversed.

Pronase Digestion and Sephadex G-50 Fractionation of Glycopeptides from Subcellular Fractions. Since the major incorporation of glucosamine is expected to be in glycoproteins, the glycopeptide pattern of cellular fractions which had been digested with the proteolytic enzyme pronase was obtained by chromatography on Sephadex G-50. The Sephadex G-50 patterns of the Pronase-digested nuclei, mitochondria, surface membrane, endoplasmic reticulum, and soluble glycoprotein fractions of ^{3}H/^{14}C mixture I (^{3}H SV-40-3T3 and ^{14}C 3T3) are shown in Figure 3. In general it can be seen that the patterns obtained for the different subcellular fractions of a particular cell line (3T3 or SV-40-3T3) are quite similar and consist of four main areas. These are (1) a sharp peak eluted at the void volume of the Sephadex column which consists of essentially undigested glycoprotein or glycopeptides of high molecular weight, (2) a smaller broader peak or shoulder immediately following the initial peak, (3) a large broad peak containing the bulk of the digested material and consisting of partially included glycopeptides, and (4) a well separated, smaller peak consisting of glycopeptides completely included in the Sephadex beads. The curves have again been normalized to a ^{3}H/^{14}C ratio of 1.00. It can be seen that in general the materials present in peaks 1 and 4 in the nuclei, mitochondria, surface membrane, and endoplasmic reticulum fractions are formed in larger amounts in 3T3 cells than in SV-40-transformed 3T3 cells. Conversely, the glycopeptides of peak 2 are present in greater amounts in SV-40-3T3 than in 3T3 cells. The most ap-

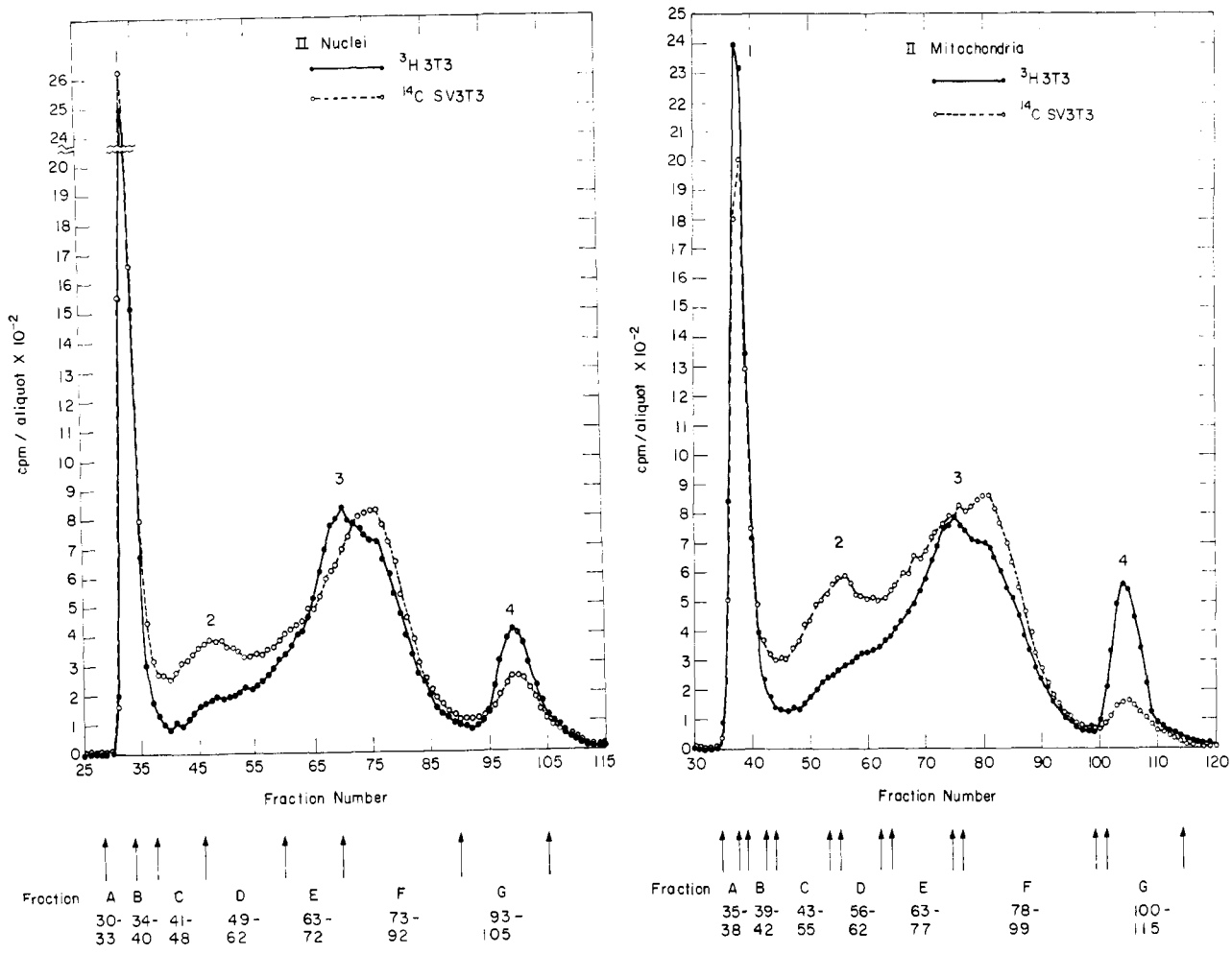

FIGURE 4: Fractionation of glycopeptides from nuclear and mitochondrial fractions on Sephadex G-50. The labeling of 3T3 and SV-40-3T3 with radioactive glucosamine was the reverse of those shown in Figure 3, namely ^{3}H-labeled 3T3 and ^{14}C-labeled SV-40-3T3 were mixed and fractionated. Following the digestion with pronase, the glycopeptides were separated into seven fractions A to G on a Sephadex G-50 column as described in the legend to Figure 3. The peaks were pooled as indicated by the arrows and were subsequently analyzed for relative amino sugar contents in each fraction (see Tables I and II).

parent differences between the glycopeptides of 3T3 and SV-40-3T3 are present in peak 3 of the nuclear and mitochondrial fractions. In both of these fractions, the peak 3 of the two cell lines are offset from each other.

Confirmation of the glycopeptide differences revealed by the Sephadex G-50 chromatography of the Pronase-digested subcellular fraction of mixture I was obtained by similar chromatography of pronase digested fractions from mixture II in which the labeling pattern was reversed. The patterns obtained for two such fractions, the nuclei and the mitochondria, are shown in Figure 4. It is apparent that these patterns are essentially identical with those obtained for the two cell lines in mixture I (Figure 3).

Comparable glycopeptide fractions from nuclei II and mitochondria II were obtained by pooling tubes from similar areas of the elution curves as shown in Figure 4. These fractions were then concentrated under nitrogen and hydrolyzed to obtain sialic acid, glucosamine, and galactosamine, which were then isolated and counted to obtain the ^{3}H/^{14}C ratios of each. As can be seen in Tables I and II, the relative amino sugar composition of glycopeptide fractions, especially fractions C and G, from SV-40-3T3 is very different from those from 3T3.

The glycopeptide pattern of a Pronase-digested mixture of ^{3}H-labeled confluent 3T3 and ^{14}C growing 3T3 nuclei on Sephadex G-50 is found to be similar to the patterns obtained for the nuclei of mixtures I and II as shown in Figure 5. In contrast to those observed in peak 3 between 3T3 and SV-40-3T3 cells, there is no apparent difference in peak 3 between growing and confluent 3T3 cells.

The glycopeptide pattern of ^{3}H-labeled ST-3T3 nuclei and ^{14}C-labeled 3T3 nuclei is shown in Figure 6. Although differences in the distribution of ^{3}H and ^{14}C radioactivities can be seen in peaks 2 and 4, there is also no apparent difference in the region of peak 3 between ST-3T3 and 3T3. Variations in the glycopeptide pattern of 3T3 from one experiment to the other may be due to differences in the extent of Pronase digestion. Comparisons between two cell lines in one experiment, however, are quite valid since digestion of both occurred as a mixture under the same conditions.

TABLE I: Relative Contents of Sialic Acid, Glucosamine, and Galactosamine in the Glycopeptide Fractions of Pronase-Digested ^{3}H 3T3/^{14}C SV-40-3T3 Mitochondria.

Glycopeptide Fractions[a]	*N*-Acetyl-neuraminic Acid/Gross	Glucos-amine/Gross	Galactos-amine/Gross
A	0.97	1.11	0.98
B	1.19	0.85	1.15
C	2.70	0.79	1.88
D	2.14	0.86	0.71
E	1.95	0.87	0.83
F	2.08	0.84	0.89
G	1.40	0.21	0.29
Before fractionation	2.44	0.65	1.51

[a] Glycopeptide fractions were obtained by Sephadex G-50 column chromatography of Pronase-treated mitochondrial fraction from ^{3}H-labeled 3T3 and ^{14}C-labeled SV-40-3T3 as described in the legend to Figure 4. The ratio of *N*-Acetylneuraminic acid/gross of a particular fraction was obtained by dividing the $^{3}H/^{14}C$ ratio of *N*-acetylneuraminic acid isolated from that fraction by the gross $^{3}H/^{14}C$ ratio of this given fraction. It can be shown that this ratio is equal to per cent of *N*-acetylneuraminic acid in ^{3}H-labeled fraction/per cent of *N*-acetylneuraminic acid in ^{14}C labeled fraction, thus representing a measurement of *relative* sialic acid contents of glycoproteins in ^{3}H- and ^{14}C-labeled fractions.

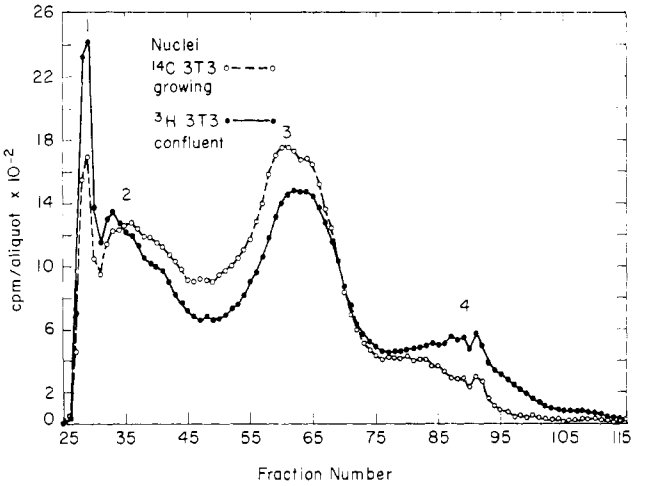

FIGURE 5: Sephadex G-50 profiles of glycopeptides from Pronase-treated nuclear fractions of [^{3}H]glucosamine-labeled confluent 3T3 and ^{14}C-labeled growing 3T3. The fractionation of the glycopeptide mixture on a Sephadex G-50 column and subsequent determination of ^{3}H and ^{14}C radioactivities were essentially the same as described in the legend to Figure 3.

Discussion

As we have mentioned in the introduction, one of the simplest explanations for the change in sugar composition following transformation of animal cells would be that exactly the same glycoproteins are formed after transformation but in different proportions. This would correspond to a decrease in normal carbohydrate-rich glycoproteins in the transformed cells which would be balanced by an increase in normal carbohydrate-poor components. Had the glycopeptide fraction obtained by Sephadex G-50 separation of Pronase-digested glycoproteins represented doubly labeled single glycopeptide species, the observed difference in the amino sugar composition of these glycopeptides would have eliminated this possibility. However, since these glycopeptide fractions probably contain more than single glycopeptide species, further studies will be required to resolve the problem.

It is possible, however, that the glycoproteins in the particulate fractions of SV-40-3T3 are actually qualitatively different from those present in 3T3 cells. Hakamori's model of incomplete antigen synthesis in the transformed cells can be regarded as a special case of this possibility. If carbohydrate chains were left incomplete it would be expected that the glycopeptide pattern for material from the transformed cells would be shifted to a lower molecular weight range. This shift may actually be seen in peak areas of Figures 3 and 4. The relatively larger amounts of material in peak 2 from transformed cells would not be anticipated from the simple interpretation of the Hakamori model. However, it may represent unique components synthesized by the transformed line. The nature of the material in the totally excluded peak is not clear and no regular variation can be seen in the amount of radioactivity found in this fraction. The possibility of differences in the degree of digestion of the membrane fractions by Bronase does not seem likely since material from the two cell lines was digested together and the recovery of material from the column had been excellent.

According to present concepts, glycoproteins and glycolipids are synthesized by the sequential addition of monosaccharides to a protein or lipid "core." The more complex glycoproteins and glycolipids contain *N*-acetylneuraminic acid,

TABLE II: Relative Contents of Sialic Acid, Glucosamine, and Galactosamine in the Glycopeptide Fractions of Pronase-Digested ^{3}H 3T3/^{14}C SV-40-3T3 Nuclei.

Glycopeptide Fractions[a]	*N*-Acetyl-neuraminic Acid/Gross	Glucos-amine/Gross	Galactos-amine/Gross
A	1.31	1.22	1.39
B	1.45	0.87	1.55
C	2.74	0.68	1.56
D	2.33	0.87	1.10
E	2.08	0.93	1.00
F	2.61	0.93	1.33
G	2.00	0.37	0.32
Before fractionation	2.20	0.68	1.38

[a] Glycopeptide fractions were obtained by Sephadex G-50 column chromatography of Pronase-treated nuclei fraction from ^{3}H-labeled 3T3 and ^{14}C-labeled SV-40-3T3 as described in the legend to Figure 4. See footnote *a* of Table I.

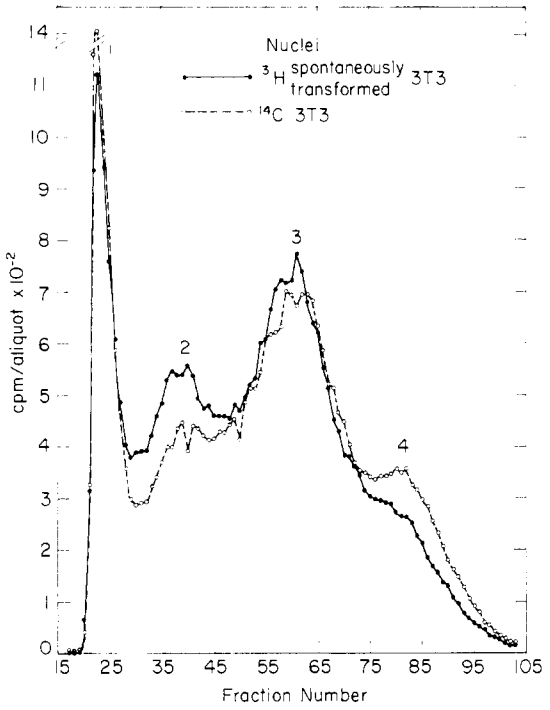

FIGURE 6: Sephadex G-50 profiles of glycopeptides from a Pronase-treated mixture of nuclei of [^{3}H]glucosamine-labeled ST-3T3 and ^{14}C-labeled 3T3.

N-acetylgalactosamine, fucose, and galactose in various branched structures on the outer parts of the molecule. The inner portions of many glycoproteins contain appreciable amounts of mannose and *N*-acetylglucosamine, while a number of complex glycolipids have a lactosylceramide base. Enzymes that are responsible for the transfer of L-fucosyl residues in the synthesis of the blood-group substances have been characterized by Shen *et al.* (1968; Kobata *et al.*, 1968). These enzymes transfer fucosyl residues from GDP-fucose to form linkages $\alpha(1\rightarrow4)$ to *N*-acetyl-D-glucosamine, or $\alpha(1\rightarrow2)$ to galactose. While the acceptor sugar and position of linkage are quite specific it is possible that the acceptor could be a small molecule, part of a glycolipid, or part of a glycoprotein. If the same principle applies to other monosaccharide transferases, it is possible that many glycoproteins and glycolipids within a cell could carry very similar patterns in their oligosaccharide side chains. This could explain the similar compositions and similar Sephadex patterns that we find for various subcellular fractions within a given cell line, although we are also aware of the possibility of cross-contamination of one fraction with another in the simple separation procedure we have followed.

This model would assume that the oligosaccharides of the major glycoproteins and glycolipids within a cell are formed by the action of the same set of transfer enzymes. These transglycosylating enzymes would use specific nucleoside diphosphate sugars as donors but would show a broad range of specificity with regard to acceptors. If these assumptions were correct, a change in the ratio of transferase enzymes following transformation would lead to similar or identical changes in the structures of all major glycoproteins and glycolipids of the cell. Each cell line would thus carry its own major "brand" of intracellular glycoprotein and glycolipid through its endowment with a set of glycosyl transferase enzymes, although some components might also be formed as a result of interaction between absolutely specific transferases and acceptors.

At present it is not possible to define the detailed structural differences between the membrane glycoproteins of transformed and nontransformed cell lines, but work along this line is continuing. From the patterns seen in the sodium dodecyl sulfate–Sephadex chromatograms it seems likely that the level of certain components in the membrane, such as peak 4, are altered rather drastically not only as a result of transformation but even as a result of the shift from the growing state to confluence. Such physiological control of membrane structure may play an important role in virus transformation and deserves further investigation.

Acknowledgments

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