allelic state of the *rel* gene. This conclusion is substantiated by our previous results that a relaxed as well as a stringent strain produces similar quantities of ppGpp during carbon source shift-down (Harshman and Yamazaki, 1971) and by exposure to hypertonic NaCl solutions (Harshman and Yamazaki, 1972). Similarly, we can conclude, from the data in Figure 1, that the *rel* gene is not necessarily involved in inhibition by levallorphan of RNA accumulation.

Although the mode of action of levallorphan is not yet fully understood, its primary effect appears to be on the permeability of *E. coli* (Simon *et al.*, 1970b). Since the strains used in the present experiment are auxotrophic for both amino acids and uracil, it is possible that levallorphan inhibits the uptake of these nutrients, which might account for the observed inhibition of growth as well as RNA accumulation. However, it is known that neither amino acid starvation nor uracil starvation causes the production of ppGpp in the relaxed strain (Cashel and Gallant, 1969; our unpublished data). On the other hand, it is possible that levallorphan causes, through its membrane effects, the inhibition of the uptake of carbon sources. The ensuing carbon source starvation would in turn result in the accumulation of ppGpp in both strains (Lazzarini *et al.*, 1971). The comparison of the data in Figure 2B and 3B may suggest that levallorphan causes a much greater inhibition of amino acid uptake in the glucose medium than in the succinate medium. The higher level of ppGpp in the stringent strain grown in the glucose medium would then reflect a response to amino acid starvation, superimposed on carbon source deprivation.

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Comparative Studies of the Carbohydrate-Containing Components of 3T3 and Simian Virus 40 Transformed 3T3 Mouse Fibroblasts†

Hisako Sakiyama and Boyce W. Burge*

ABSTRACT: Glucosamine-labeled glycoproteins and glycopeptides of normal and SV-40 transformed 3T3 mouse fibroblasts have been analyzed by a combination of acrylamide gel electrophoresis and chromatography on Bio-Gel P-10. Controls were included for the possible influence of cell growth rate on these measurements. Though the normal and transformed cell lines examined have demonstrable differences in overall carbohydrate content, the glycoproteins and glycopeptides of a bulk membrane fraction of the two lines could not be distinguished by comparison of profiles of glycoproteins on acrylamide gel electrophoresis or by profiles of glycopeptides on Bio-Gel P-10 chromatography. Elution of well-defined glycoprotein peaks from acrylamide gels, followed by chromatography of glycopeptides from the peak fraction also failed to reveal differences. Several transformation mechanisms are suggested which would result in cells of altered carbohydrate content but with similar or identical glycoproteins and glycopeptides.

Transformed cells are characterized by loss of growth control(s) present in normal cells. This loss of control leads *in vivo* to metastasis and invasive and unlimited growth. *In vitro* transformed cells exhibit failure of contact inhibition and thus pile up in multilayered growth. These characteristics of transformed cells may well depend on chemical alterations present in malignant cell membranes. For instance a purified glycoprotein (wheat germ agglutinin) aggregates transformed cells but not normal or nontransformed cells (Burger and

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Goldberg, 1967). It was postulated that an agglutination site containing *N*-acetylglucosamine (Jansons *et al.*, 1970) is exposed in transformed cell membranes but not in normals. This phenomenon suggests a possible alteration in the carbohydrate-containing molecules of the cell surface.

The carbohydrate content of cell membranes has been examined by several groups. Ohta found that transformed cells have a reduced amount of sialic acid compared to normal cells (Ohta *et al.*, 1968). Wu reported a pronounced decrease in most neutral and amino sugars, particularly in the sialic acid and *N*-acetylgalactosamine of the membrane of SV40-transformed 3T3 cells, compared to normal cells from which the transformed cells were derived (Wu *et al.*, 1969). Buck also showed that BHK21/13 cells transformed either by SV40 or polyoma virus have decreased amounts of fucose, mannose, and galactose per milligram of cell protein (Buck *et al.*, 1970a).

Changes in glycosyl transferase activities seem also to be involved in the carbohydrate-related changes seen in transformed cells. Grimes has investigated 3T3 mouse fibroblasts and several virus-transformed derivatives. Transformed cells that had only 60% of the normal level of sialic acid (expressed as micrograms of sialic acid/milligram of protein) were found to have sialyl transferase specific activities that were only 55–60% those of normal cells. These characteristics were found not to be related to the rate of growth of the cells (Grimes, 1970).

These results suggested to us that a high-resolution study of the glycoproteins and glycopeptides of transformed cells should reveal the molecular basis of gross changes in cell carbohydrate content. The results reported here indicate that the alteration typical of transformed cells is a decrease in the amount of glycoprotein present per cell, with almost no resolvable differences in the gel profiles of either glycoproteins or glycopeptides.

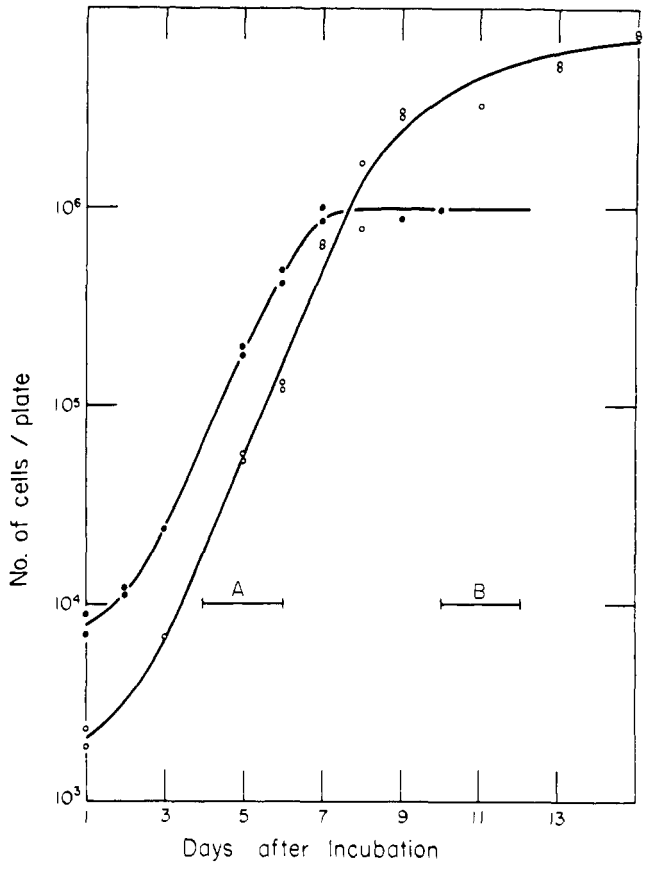


FIGURE 1: Growth condition of cells during labeling. 3T3 (●—●) and SV3T3 cells (○—○) were labeled at cell densities indicated by solid lines. Dividing 3T3 cells were labeled during logarithmic growth (A) while nondividing 3T3 cells and SV3T3 cells were labeled after they had reached confluency (B).

Experimental Procedures

Cell Cultures. The 3T3 cell line of Swiss mouse embryo fibroblasts (Todaro and Green, 1963) and SV40-transformed 3T3 cells (SV-3T3) (Black, 1966) utilized in these experiments were obtained from Dr. Paul Black. Cells used in this report were grown in Eagle's minimal essential medium with four times the concentration of vitamins and amino acids, 10% fetal calf serum, and 50 μg and 75 units per ml of streptomycin and penicillin, respectively. Cells were grown in roller bottles, in Blake bottles, or in plastic bottles (Falcon). The saturation density of 3T3 cells was 1×10^6 cells per 5-cm plastic petri dish at the beginning of the experiment, but the density reached 4×10^6 cells per plate after cells had been passaged 3 months. Cells were used at the middle of this period, therefore the saturation density was between 1×10^6 and 4×10^6 cells per plate. The saturation density of SV-3T3 cells was 10×10^6 cells per 5-cm plastic petri dish.

Labeling and Subcellular Fractionation. 3T3 and SV-3T3 cells were labeled at cell densities indicated by the solid lines in Figure 1. Dividing 3T3 cells were labeled during logarithmic growth (Figure 1A), and nondividing 3T3 cells and SV-3T3 cells were labeled after they had reached confluence (Figure 1B). Dividing 3T3 cells were labeled with D-[6-^{3}H]-glucosamine or with ^{14}C-labeled amino acids (reconstituted protein hydrolysate, Schwarz BioResearch), nondividing 3T3 cells were labeled with D-[1-^{14}C]glucosamine or [^{3}H]amino acids and SV-3T3 cells were labeled with D-[6-^{3}H]glucosamine or [^{14}C]amino acids for 2 days. When cells were harvested the medium was saved and the monolayer was washed with phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954). Cells were then incubated in TNE buffer[1] for 5 min at 37°. EDTA is known to solubilize certain proteins of red blood cell membrane (Rosenberg and Guidotti, 1969). This treatment completely removes the SV-3T3 cells from the glass surface. Normal cells remaining on the glass surface at the end of this treatment were removed by gentle pipetting. Cells were then pelleted at 500*g* for 10 min. The pellet was resuspended in cold reticulocyte standard buffer (RSB) and homogenized in a glass Dounce honogenizer until more than 80% of cells are broken. Nuclei and unbroken cells were separated by centrifugation at 600*g* for 10 min. Resuspended nuclei and supernatant were layered separately on discontinuous gradients of sucrose in TNE buffer. Nine milliliters of 44% (w/w) sucrose was placed at the bottom of the centrifuge tube (Beckman SW27 rotor, 40 ml/tube), then 11 ml of 40% and 16 ml of 24% sucrose were layered carefully above. Finally, 4 ml of sample was layered on the top of the sucrose. The discontinuous sucrose gradient was centrifuged at 25,000 rpm for 3 hr at 4°. Approximately 1 mg of protein was layered on each gradient. The resulting bands were removed with a Pasteur pipet and stored at −20° for later analysis. This fractionation procedure is made graphic in Figure 2. Protein of each fraction was determined by the method of Lowry

[1] Abbreviations used are: TNE, 0.5 M Tris–0.1 M NaCl–0.01 M; EDTA, pH 7.4; RSB, reticulocyte standard buffer.

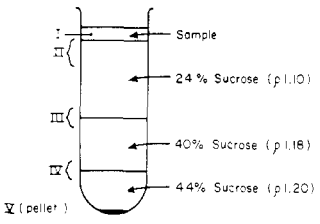


FIGURE 2: Distribution of cell components on discontinuous density gradients of sucrose following equilibrium centrifugation.

(Lowry *et al.*, 1951) using bovine serum albumin as standard. Medium and EDTA extracts were centrifuged at 8000*g* for 15 min to remove cells and cell debris and then frozen at −20°.

Preparation of Samples for Gel Electrophoresis. Medium and EDTA extracts were exhaustively dialyzed against 0.05 M sodium phosphate buffer (pH 7.0) at 4°. Particulates were disrupted by treatment with 2% sodium dodecyl sulfate–0.5 M urea–100 mM dithiothreitol, heated at 90° for 5 min, and followed by dialysis against a solution of 15% sucrose, 1% sodium dodecyl sulfate, 1% mercaptoethanol, and 0.005 M sodium phosphate (pH 7.0) for 3 hr.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels were prepared according to the methods described by Summers (Summers *et al.*, 1965) with some modification (Strauss *et al.*, 1968). All gels used were 7.5% acrylamide. For the determination of approximate molecular weight, Sindbis virus glycoproteins were used as markers (Burge and Strauss, 1970). Tritium and ^{14}C radioactivity was determined in a three-channel Packard scintillation counter, using a Triton X-100–toluene scintillation solution (Patterson and Greene, 1965).

Elution of Glycoproteins from Gels (Weber and Osborn, 1969). Gel slices were crushed with a tight Dounce homogenizer. The gel material was suspended in 1 ml of 0.1% sodium dodecyl sulfate solution and kept for several hours at 37°. The solution was withdrawn and another 1 ml of 0.1% sodium dodecyl sulfate was added. Elutions were combined and 0.1 ml of eluents from each slice were counted. Elution efficiency was about 80%. Glycoproteins from certain peaks were pooled, dialyzed against distilled water overnight, lyophilized, and resuspended in 0.1 M sodium phosphate buffer (pH 7.8) containing 0.001 M $CaCl_2$ for Pronase digestion.

Pronase Digestion of Glycoproteins and Bio-Gel P-10 Fractionation. Pronase digestion was carried out according to the procedure of Spiro (1965). All samples used for Pronase treatment were dialyzed against distilled water, lyophilized, and resuspended in 0.1 M sodium phosphate buffer (pH 7.8), containing 0.001 M $CaCl_2$, and Pronase (Calbiochem B grade) was added initially to equal 1% of the sample protein by weight. A few drops of toluene were added to prevent bacterial growth. Mixtures were incubated at 37° for 5 days, with daily additions of Pronase to a final concentration of 5%. At the end of the incubation, insoluble material was removed by centrifugation and supernatant fluids were analyzed on Bio-Gel P-10 (100–200 mesh, Bio-Rad Laboratories Richmond, Calif.). Chromatography was carried out on a 1.1 × 55 cm column which was eluted with 0.1 M sodium phosphate buffer (pH 7.8). The sample of about 1 ml was placed on the column and fractions of approximately 1 ml were collected and counted. Bio-Gel P-10 excludes molecules larger than 12,000. Molecular weights of cell membrane glycopeptides were estimated by methods described by Burge and Strauss (1970) using sucrose, and glycopeptides of fetuin and Sindbis virus as markers. It was assumed that Pronase digestion yields glycopeptides which are 70–80% carbohydrate by weight.

Determination of Relative Sialic Acid Content in Various Membrane Glycopeptides. Glycopeptides were hydrolyzed with 0.1 N sulfuric acid at 80° for 1 hr. After hydrolysis they were analyzed by Bio-Gel P-10 chromatography. A sialic acid marker was found to elute several fractions ahead of sucrose, a molecule of the same molecular weight. In expressing sialic acid associated counts as a fraction of the total counts on the Bio-Gel, the counts in the void volume (chiefly mucopolysaccharide) were not included (see Table III). As sialic acid is derived intracellularly from glucosamine (Warren, 1966) it was assumed that during a labeling period of 48 hr the glucosamine and sialic acid of all glycoproteins would approach the same (maximum) specific activity, and that use of radioactivity to determine relative amounts of the two sugars would be valid.

Results

Distribution of Incorporated Label in Cell Fractions. Cultures of logarithmically dividing and nondividing 3T3 cells and SV-3T3 cells were labeled with radioactive amino acids or glucosamine as described in Methods. Glucosamine (35–45%) and amino acids (5–10%) were incorporated into cells during the 2-day labeling period. Of the labeled glucosamine incorporated by transformed cells, 40% was excreted into the medium, while normal cells excreted 70–80%. The greater part of this excreted material was mucopolysaccharide as was shown both by its susceptibility to hyaluronidase and by its solubility in trichloroacetic acid. The percentage of excreted radioactivity found in mucopolysaccharide was 83, 73, and 42% in dividing, nondividing, and SV-3T3 cell medium, respectively. Approximately 20% of the radioactive amino acids taken up by cells were excreted into the medium in all three cases.

The cells labeled with either amino acids or glucosamine were washed once with PBS and then incubated in EDTA-containing buffer (saline buffer with 10 mM EDTA; Methods) for 5 min at 37°. By this treatment about 3–4% of trichloroacetic acid precipitable glucosamine label was released from the cell surface without decreasing cell viability (ability to exclude colloidal dyes such as erythrocine B). Incubation for up to 15 min did not increase significantly the radioactivity released by EDTA.

After extraction with EDTA, cells were homogenized, and the particulate components were separated on a discontinuous sucrose gradient into five fractions (Figure 2). The distribution of acid-precipitable radioactivity and protein in fractions from nondividing 3T3 cells in shown in Table I. Both smooth and rough cell membrane, including the plasma membrane, are found in fraction III which includes all rapidly sedimenting structures of density greater than 1.10 g/cm^3 and less than 1.18 g/cm^3. This fraction will be called the membrane fraction. Fraction V contains nuclei and unbroken cells. Twenty per cent of the total protein and 50% of the glucosamine label were found in the membrane fraction. As would be expected the specific activity of the amino acids label (counts per minutes per microgram of protein) was similar for all fractions. The specific activity of glucosamine in the membrane fraction was always greater than other fractions by a factor of 3 to 5. The same qualitative results were obtained with dividing 3T3 and SV-3T3 cells.

Acrylamide Gel Electrophoretic Analysis of Excreted, EDTA

TABLE I: Distribution of Radioactivity and Protein in Fractions of Confluent 3T3 Cells.[a]

Fractions	Per Cent of Total Label		Counts per Minute per μg of Protein		
	[^{14}C]-Glucosamine	^{3}H Amino Acids	Per Cent of Total Protein	[^{14}C]-Glucosamine	^{3}H Amino Acids
I	7.3	19	22.6	1100	1000
II	30.9	49.4	49.2	1500	1100
III	52.9	20.3	15.5	6200	1800
IV	1.7	1.8	4.8	1300	
V	7.7	9.5	8.4	2000	1400

[a] Following equilibrium centrifugation of discontinuous sucrose gradients the resulting bands were carefully removed with a pasteur pipette. Small amounts of each fraction were precipitated with trichloroacetic acid and resuspended in 0.1 N NaOH for determination of protein and radioactivity.

Released, and Membrane Fraction. The three sugar-containing molecules of animal cells (mucopolysaccharide, glycoprotein, and glycolipid) can be separated conveniently by the sodium dodecyl sulfate–phosphate acrylamide gel electrophoresis method (Summers *et al.*, 1965). Glycoproteins are found throughout the gel, according to their molecular weight, except for glycoproteins containing a very large fraction of their mass as carbohydrate. These will move at an anomalous position (Bretscher, 1971). Mucopolysaccharides, chiefly hyaluronic acid (Hamerman *et al.*, 1965), remain at the top of the gel due to their high molecular weight (2×10^5 to 2×10^6) and lack of affinity for sodium dodecyl sulfate. Glycolipids are found to move rapidly toward the anode, in the region of the Bromophenol Blue dye marker (Prival[2]). Therefore, we used this method to provide high resolution of glucosamine-containing glycoproteins and to monitor glucosamine-labeled mucopolysaccharide and glycolipid simultaneously. The profiles from gel electrophoresis of excreted, EDTA extract, and membrane fractions are seen in Figure 3. Amino acid label and glucosamine label were coelectrophoresed for each of the 3 samples to allow comparison of protein and carbohydrate peaks.

Excreted Material. In Figure 3A, the glucosamine-labeled component of the excreted medium is seen to consist chiefly (80%) of high molecular weight material which penetrates

[2] Joan Prival, unpublished data.

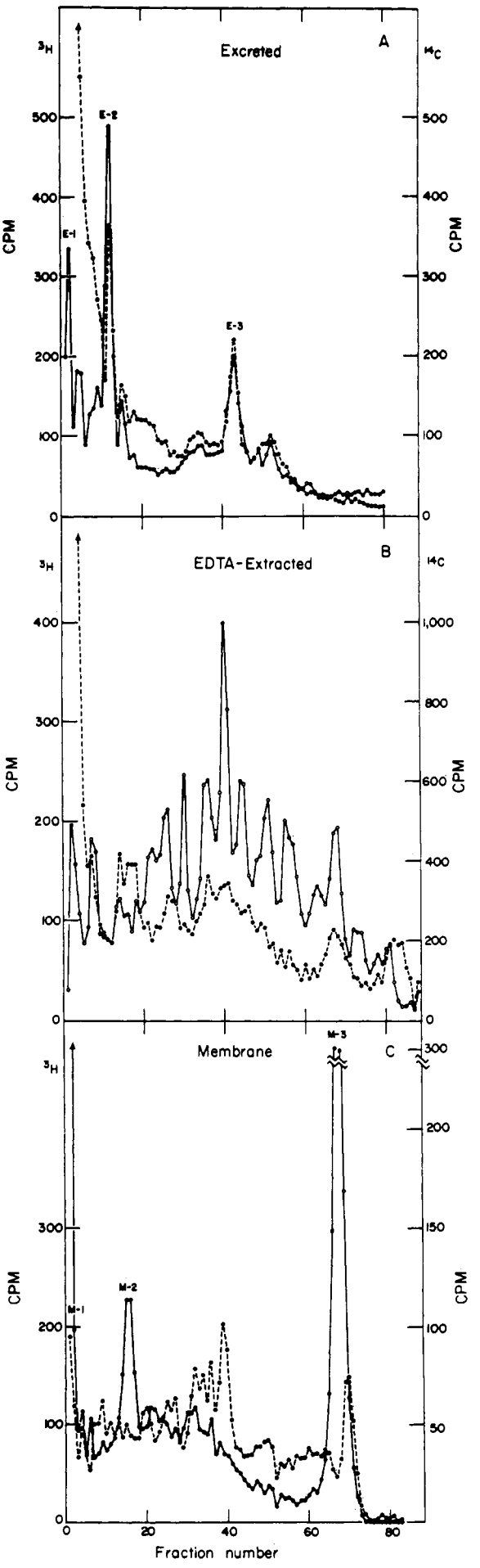
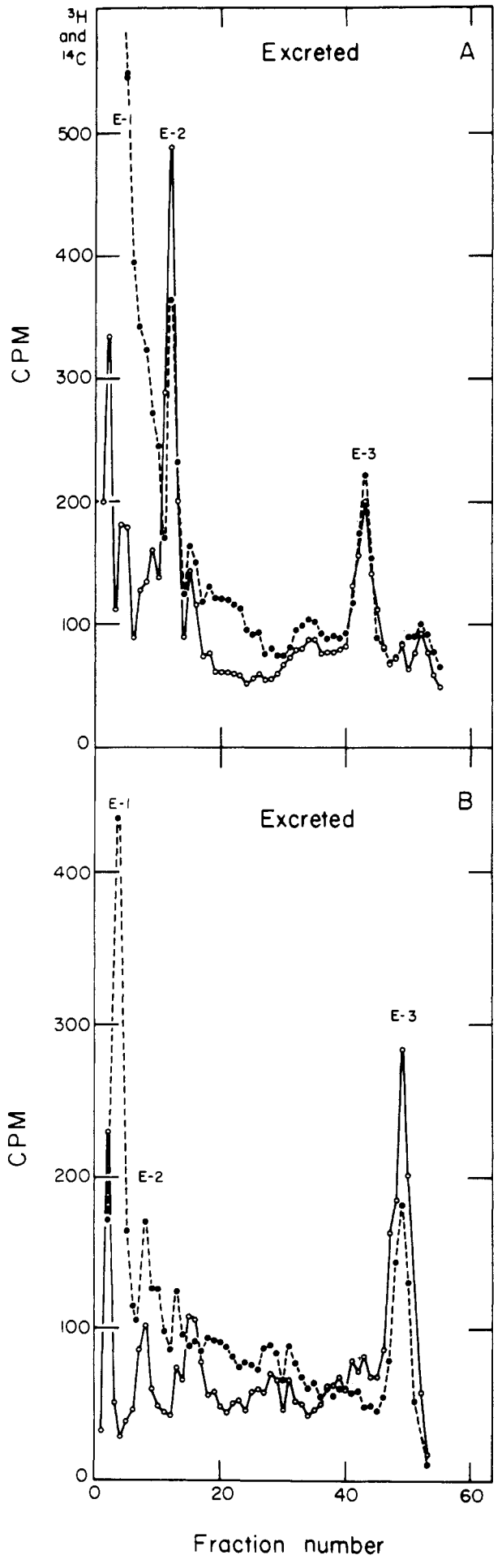


FIGURE 3: Analysis of glycoproteins and proteins on polyacrylamide gels. Excreted EDTA extract and membrane fractions doubly labeled with glucosamine and amino acids were solubilized with 2% sodium dodecyl sulfate–0.5 M urea–100 mM dithiothreitol and heated at 90° for 5 min. Samples were analyzed on polyacrylamide gels. Gels were 7.5% acrylamide, 9 cm in length and 0.6 cm in diameter. Electrophoresis was for 15 hr at 3 mA per gel at room temperature. Migration is from left to right. A: Excreted materials from dividing cells (●- - -●- - -●) [^{3}H]glucosamine, (○—○) ^{14}C amino acids; B: EDTA extract from dividing cells (●- - -●- - -●) [^{3}H]glucosamine, (○—○—○) ^{14}C amino acids; C: membrane fractions from nondividing cells (○—○) [^{14}C]glucosamine, (●- - -●- - -●) ^{3}H amino acids.



only the first few milimeters of the gel. It seemed probable that this material was mucopolysaccharide, probably hyaluronic acid, which is known to be excreted by fibroblasts in culture (Grossfeld *et al.*, 1955). We tested this possibility by digesting medium exhaustively with chromatographically purified hyaluronidase for 5 days (Weissmann *et al.*, 1954). After digestion the medium was dialyzed against distilled water, followed by usual procedure for gel analysis. By this treatment the amount of radioactivity migrating in this area was reduced from 80% to about 4% of the total counts. The medium was also treated with cetylpyridinium chloride (Knecht *et al.*, 1967). After the removal of cetylpyridinium chloride precipitable material from the medium sample the supernatant was examined by acrylamide gel electrophoresis. Peak 1 had disappeared completely. These results suggest that peak 1 of medium is chiefly hyaluronic acid. Peak 1 of the membrane fraction is also hyaluronic acid.

Beyond the first few fractions of this gel, amino acid and glucosamine labeled peaks are seen to coincide, suggesting that aside from hyaluronic acid, most of the labeled glucosamine is present as glycoprotein. A few glucosamine counts were found in the position where glycolipids run, near the dye marker.

EDTA Extract and Membrane Fraction. The gel profiles of EDTA extract (Figure 3B) and membrane fraction (Figure 3C) are chiefly remarkable in that their profiles do not resemble each other; apparently the EDTA extraction removes a unique fraction of glycoprotein molecules from the cell surface. Both membrane and EDTA glucosamine labeled profiles include a small amount of peak 1, presumably hyaluronic acid, with little associated amino acid label. The amino acid profile of the EDTA extract has about 15 well-defined peaks. A relatively large amount of glucosamine label (20%) is found in the glycolipid region of the membrane fraction profile, confirming the expectation that this fraction, which includes plasma membrane, should also include considerable glycolipid.

Glycoprotein Peaks E-2 and M-2. Both the excreted fraction and membrane fraction are found to have a prominent glucosamine labeled peak between fractions 10 and 20 (Figure 3A,C) which we have designated peaks E-2 and M-2, respectively. These peaks are not of identical molecular weight. Comparison of samples by coelectrophoresis reveals that peak E-2 has the greater molecular weight (nominally ~150,000) while peak M-2 has a nominal molecular weight of ~120,000 (Table II). Because of the uncertain effects of carbohydrate on mobility of glycoproteins, only nominal values are quoted for molecular weight. The very sharp prominent peak 3 of the membrane fraction is chroloform–methanol extractable, and therefore this peak is presumed to be glycolipid.

Comparison of Excreted Material from Normal and Transformed Cells. Figure 4 compares media from dividing 3T3 and SV-3T3 cells which were grown in the presence of [^{3}H]-glucosamine and [^{14}C]amino acids. The medium of SV-3T3 cells is lacking a prominent peak E-2. The glucosamine-labeled radioactivity found in peak E-1 of normal cells accounts

FIGURE 4: Comparison of excreted materials from normal dividing cells and SV3T3 cells. Glucosamine and amino acids labeled excreted fractions were mixed and coelectrophoresed on acrylamide gels. Gels were 7.5% acrylamide, 9 cm long. A: Excreted materials from dividing 3T3 cells (●- - -●- - -●) [^{3}H]glucosamine (○—○—○) ^{14}C amino acids, B: excreted materials from SV3T3 cells (● - -●--●) [^{3}H]glucosamine (○—○—○) ^{14}C amino acids.

TABLE II: Nominal Molecular Weight of Peak 2 from Excreted, EDTA Extract, and Membrane Fraction.[a]

	R_F Related to Dye[b]	Nominal Mol Wt
Membrane (M-2)	0.173	~120,000
EDTA extract	0.215	~130,000
Excreted (E-2)	0.246	~150,000

[a] To determine approximate molecular weight of peaks of three fractions, Sindbis virus proteins were used as markers (Strauss *et al.*, 1968). [b] Bromophenol Blue.

for about 80% of all counts in the gel, while the E-1 peak of transformed cells accounts for only 40%. This result agrees with earlier data that transformation of cells by SV40 leads to marked diminution in rate of mucopolysaccharide synthesis (Hamerman *et al.*, 1965). Proteins and glycoprotein profiles from nongrowing cells were not distinguishable from those of growing cells.

Comparison of Membrane Fraction of Normal and Transformed Cells. Glycoproteins of the membrane fraction from nondividing normal cells and SV-3T3 cells were compared on acrylamide gel in Figure 5. The profiles are complex, indicating the presence of a great many species of membrane glycoprotein but no significant difference was found in the glycoprotein region of these profiles (Fraction 5–70). Glycoproteins of the membrane fraction from growing normal cells showed a profile indistinguishable from these shown in Figure 5.

Comparison of Glycopeptides of Membrane Fraction and EDTA Extract. It seemed to us to be conceivable that the altered carbohydrate content of virus-transformed cells might be explained in part by alteration of their glycopeptides, perhaps by a decrease in chain length. Thus we examined glycopeptides by molecular sieving on Bio-Gel P-10, a method which is chiefly sensitive to changes in molecular weights (although anions are retarded slightly relative to neutral molecules of the same weight).

Samples of EDTA extract and of the membrane fraction were digested extensively with Pronase and prepared for chromatography on Bio-Gel P-10 as described in Methods. In general the patterns consist of two parts. The sharp peak which eluted at the void volume of Bio-Gel P-10 consists principally of mucopolysaccharides, but also of glycopeptides of high molecular weight and undigested glycoproteins. Glycopeptides of molecular weight less than ~12,000 are retarded by the gel and appear, in general, as a broad peak eluting after the void volume.

The Bio-Gel P-10 patterns of the Pronase-digested EDTA extracts from dividing 3T3, nondividing 3T3, and SV-3T3 cells are shown in Figure 6. The glycopeptide profile of dividing cells has a broad major peak centered at a position corresponding to a molecular weight of 3300; this peak is broader than that of nondividing cells (Figure 6A). It has a shoulder on the low molecular weight side of the peak. Glycopeptides of nondividing cells have a rather symmetrical narrow peak. These results suggest that dividing cells have a more heterogeneous size distribution of glycopeptides in the EDTA extract than do nondividing cells.

The glycopeptide profile of SV-3T3 cells is rather similar to that of nondividing cells, but has in addition a shoulder

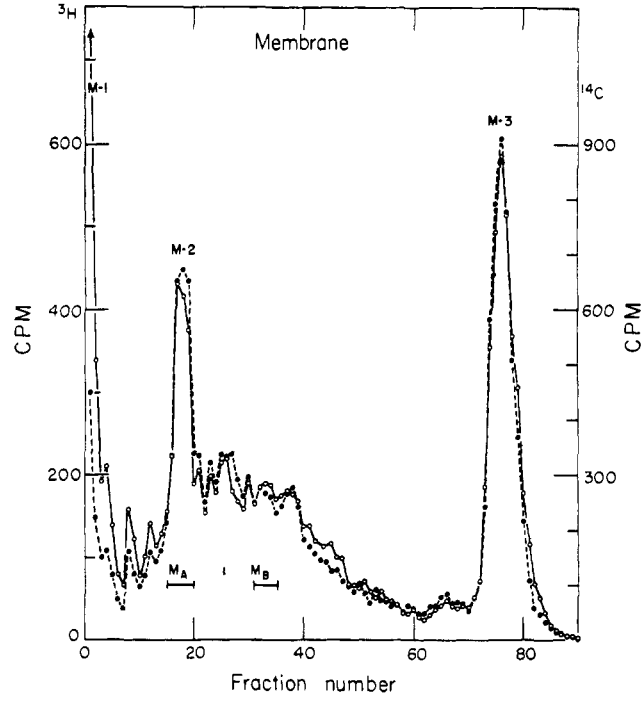


FIGURE 5: Comparison of membrane fraction of normal and transformed cells. 3T3 and SV3T3 cells were labeled with [^{14}C]glucosamine and [^{3}H]glucosamine, respectively. After the fractionation of cells on discontinuous sucrose gradients, membrane fractions were mixed and applied to an acrylamide gel. (●- - -●- - -●) [^{3}H]Glucosamine; (O—O—O) [^{14}C]glucosamine.

migrating on the high molecular weight side of the main peak. However, no shift to a lower average molecular weight was seen. Though not shown here, direct comparison of glycopeptide fractions of dividing 3T3 cells and SV-3T3 cells showed identical profiles.

It is possible, by an extension of the Bio-Gel procedure, to estimate the relative sialic acid content of two glycopeptide populations. To do this glycopeptides were hydrolyzed with 0.1 N H_2SO_4 at 80° for 1 hr, a procedure that removes sialic acids, but not other sugars, from the glycopeptide. After hydrolysis the samples were chromatographed on Bio-Gel P-10 (Figure 6C). The third peak which elutes 2 or 3 fractions ahead of sucrose comigrates with sialic acid marker. After the removal of sialic acid the main peaks became less broad and they were shifted to a smaller average molecular weight. The shoulder leading the main glycopeptide peak of SV-3T3 cells became more marked, but the average molecular weight of glycopeptides minus sialic acid was identical for normal and transformed cells. We found no significant difference in the relative labeled sialic acid content of normal and transformed cells.

The glycopeptide patterns of the membrane fraction from dividing 3T3, nondividing 3T3, and SV-3T3 cells are shown in Figure 7. The glycopeptide profile of dividing cells is shifted toward higher molecular weight (Figure 7A) but after the removal of sialic acid, the shift was no longer found (Figure 7B). This difference in profile prior to hydrolysis is reflected in the greater amount of sialic acid released from glycopeptides of dividing cells (Table III). The profiles of nondividing and SV-3T3 cells look quite similar both before and after hydrolysis (Figure 7C,D), direct comparison of growing 3T3 and SV-3T3 cells (not shown here) also revealed very similar profiles.

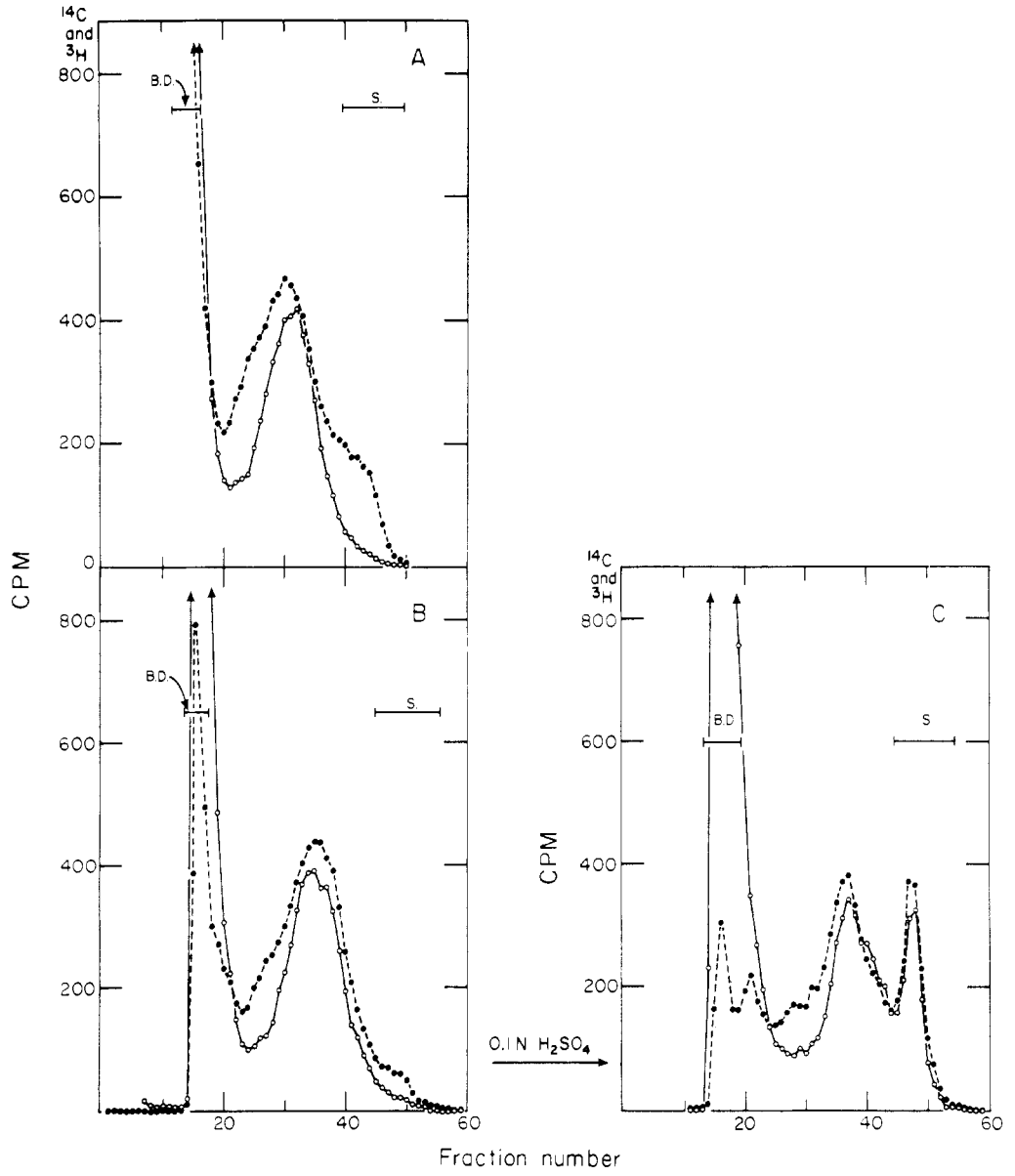


FIGURE 6: Comparison of EDTA-extracted glycopeptides on Bio-Gel P-10. Dividing 3T3 and SV3T3 cells were labeled with [^{3}H]glucosamine and nondividing 3T3 cells were labeled with [^{14}C]glucosamine. EDTA extracts were digested with Pronase and the resulting glycopeptides were fractionated on a Bio-Gel P-10 column (1.1 × 55 cm). The column was eluted with 0.1 M sodium phosphate buffer, pH 7.8. Fractions of 1 ml were collected and counted. For the determination of relative sialic acid content, samples were hydrolyzed with 0.1 N sulfuric acid at 80° for 1 hr. (●- - -●- - -●) [^{3}H]Glucosamine; (○—○—○) [^{14}C]glucosamine. A and B: before hydrolysis C: after hydrolysis, B.D.: Blue dextran, S: sucrose.

In both this study and that of Meezan *et al.* (1969) there appears to be a slight shift toward higher molecular weights of transformed cell glycopeptides as compared to normal 3T3 glycopeptides. This is illustrated with very high resolution in Figure 4 of Meezan *et al.* (1969) where peak 2 glycopeptide material is in excess in transformed cells compared to normal cells. On Bio-Gel P-10, peaks 2 and 3 of Meezan *er al.* appear as a single peak, the major glycopeptide peak, and the increase of material in peak 2 is reflected in a shift of the main glycopeptide peak of transformed cells to slightly higher average molecular weight (*e.g.*, Figures 6 and 7). The significance of this shift as a consequence of transformation is made doubtful by the occurrence of a similar shift seen when growing and nongrowing "normal" 3T3 cells are compared (also Figures 6 and 7).

Glycopeptides of Glycoprotein Fractions of Discrete Molecular Weight. As shown in Figures 6 and 7, glycopeptide peaks of both cell membranes and EDTA extracts are broad. This suggests the presence of many glycopeptide species of various molecular weights. Moreover no significant difference in profiles of glycopeptides was found in the comparison of normal and transformed cells.

There are no data as yet to suggest whether each membrane glycoprotein has a single or several glycopeptide species, or what range of glycopeptide sizes and composition are to be expected in membrane glycoprotein. We therefore examined the glycopeptides of a limited molecular weight spectrum of glycoproteins, to determine whether a simplified glycopeptide profile might be obtained, and whether a simplified profile would allow us to detect differences between the normal and transformed cells.

With these objects in mind we examined two regions of the

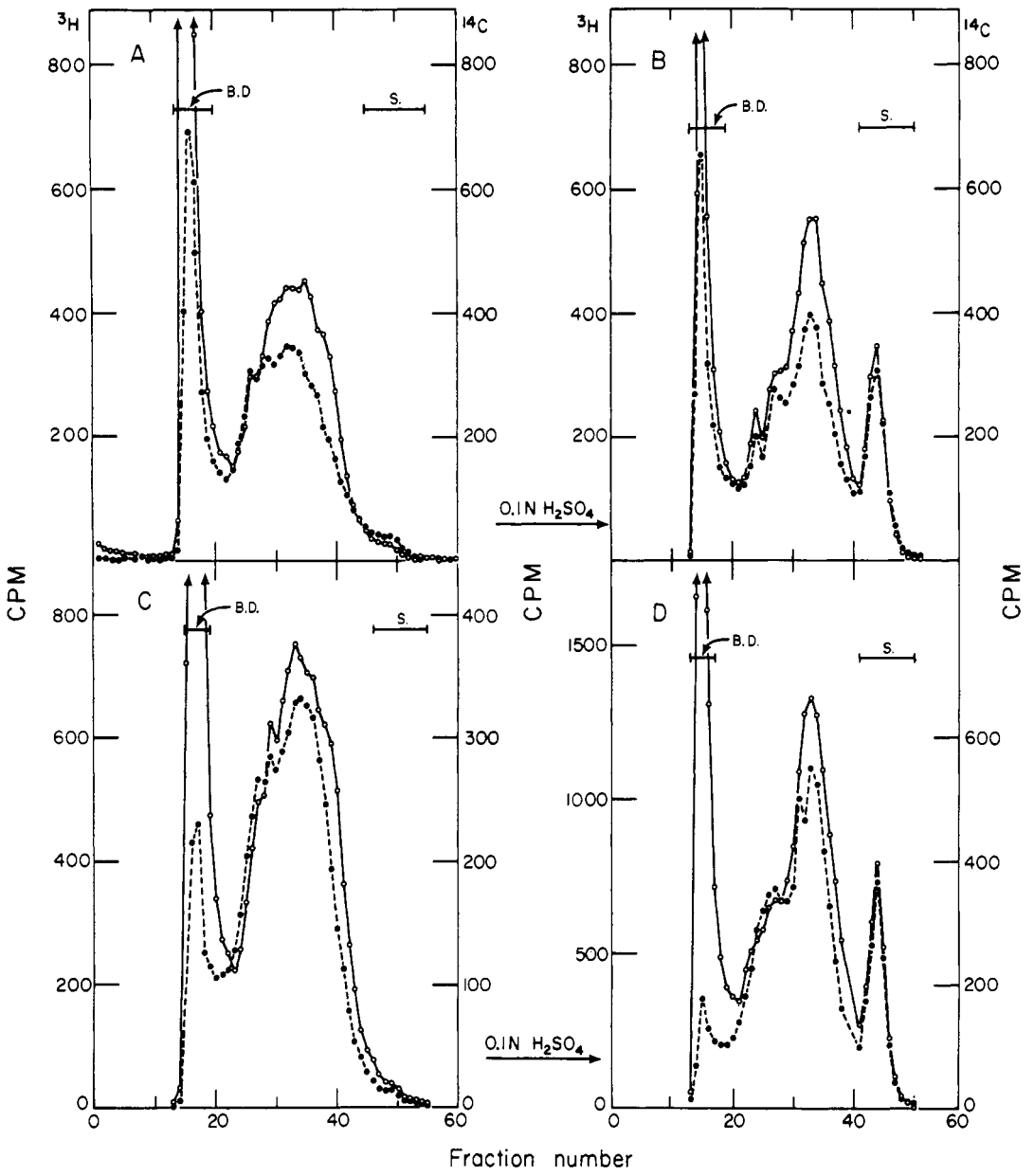


FIGURE 7: Comparison of glycopeptides from membrane fractions. Dividing 3T3 and SV3T3 cells were labeled with [^{3}H]glucosamine and non-dividing 3T3 cells were labeled with [^{14}C]glucosamine. (●- - -●- - -●) [^{3}H]Glucosamine; (○—○—○) [^{14}C]glucosamine. A and C: before hydrolysis with sulfuric acid, B and D: after the hydrolysis. B.D.: Blue dextran, S: sucrose.

acrylamide gel profile of membrane fractions of normal and transformed cells described previously in Figure 5. The first region M_A (fraction 15–20, mol wt ~120,000) corresponds to the apparently pure glycoprotein peak M-2, while the second region M_B (fraction 31–35, mol wt ~60,000) is from an area in which there are no well-defined peaks. Glycoproteins from these regions were eluted from the gel, digested with Pronase, and compared on Bio-Gel P-10 (Figure 8). The peaks seen were much narrower than those of unfractionated glycoproteins (*e.g.*, Figures 6 and 7): in particular, the glycopeptides derived from membrane peak M-2 were very homogeneous, with an approximate molecular weight of 3600 (M_A Figure 8A). The glycopeptide from the M_A region is as homogeneous, in terms of peak width, as that from a pure glycoprotein, that of Sindbis virus (Burge and Strauss, 1970). The M_B region of the acrylamide gel yielded a more complex pattern but showed a distinct shoulder on the lower molecular weight side of the profile (Figure 8C). The main peak 2 in the profile is also of considerably higher molecular weight (~5400) than that of peak 2 in Figure 8A, and is clearly a different glycopeptide species.

Peak 1 of 8C, is presumably a large glycopeptide, of molecular weight greater than 10,000 and it is present in relatively greater amounts in the transformed cells. If the peak were simply the result of incomplete Pronase digestion, one would expect to find the same relative amounts in normal and transformed cell fractions. Therefore, this glycoprotein fraction is apparently not identical in normal and transformed cells. Aside from the differences in amounts of peak 1, the glycopeptides of normal and transformed cells do not differ, even with the described scheme for improved resolution. In addition there is no difference in the relative sialic acid content of glycopeptides from the normal and transformed cells (Table III). Figure 8 shows that Bio-Gel elution profiles of glycopeptides from unique size classes of glycoprotein differ, suggesting that several differentiable glycopeptides are found in

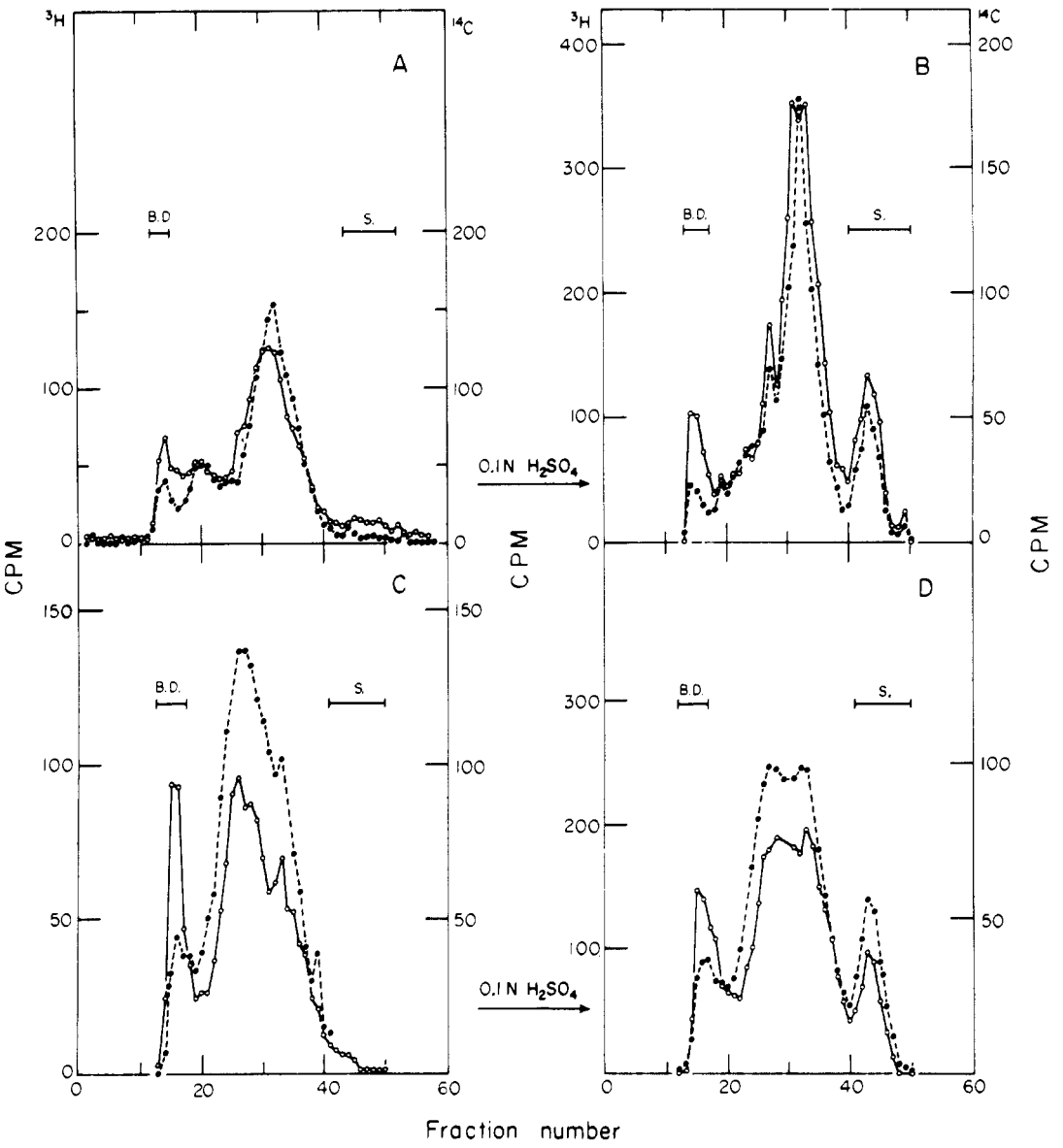


FIGURE 8: Comparison of glycopeptides from glycoprotein fractions of discrete molecular weight. Glycoproteins of membrane fractions were eluted from acrylamide gels (M_A and M_B of Figure 5) and digested with Pronase. Glycopeptides were fractionated on Bio-Gel P-10. (●--●--●) [^{3}H]Glucosamine (SV3T3); (○—○—○) [^{14}C]glucosamine (3T3). A and C: before acid hydrolysis, B and D: after hydrolysis. B.D.: Blue dextrane, S: sucrose.

TABLE III: Relative Contents of Sialic Acid in the Glycoproteins of Various Fractions.[a,b]

	Dividing 3T3	Non-dividing 3T3	SV3T3
EDTA extract		25.8	26.6
Membrane fraction	21.8	17.2	17.3
M_A (Figure 5)		17.5	15.8
M_B (Figure 5)		14.9	15.3

[a] Cpm in peak 3/(cpm in peak 2 + peak 3) × 100. [b] Glycopeptides were hydrolyzed with 0.1 N sulfuric acid at 80° for 1 hr. After hydrolysis samples were subjected to Bio-Gel P-10 chromatography. The sum of sialic acid counts was divided by total counts excepting those counts in the void volume.

cell membranes associated in a specific way with certain glycoproteins.

Relation of Glycoprotein Molecular Weight to Glycopeptide Profiles. It was of interest to extend the experiments on the relation of glycopeptide size and heterogeneity to the molecular weight of the glycoprotein from which they are derived. Glycoproteins of the membrane fraction from dividing and nondividing cells labeled with either [^{3}H]glucosamine or [^{14}C]glucosamine were run separately on acrylamide gels. Peak M-2 glycoproteins of nondividing cells were combined with glycoproteins from fraction 26–30 or 45–50 of dividing cells. Glycoproteins of these regions have molecular weights, respectively of 120,000, 60,000, and 35,000. They were analyzed on Bio-Gel P-10 following exhaustive Pronase digestion. The results are shown in Figure 9. Peak M-2 glycoproteins have homogeneous glycopeptides of molecular weight ~3600. In contrast glycoproteins from fractions 26–30 and 45–50 have profiles compatible with the presence of several different sizes of glycopeptides. For example in the elution pattern

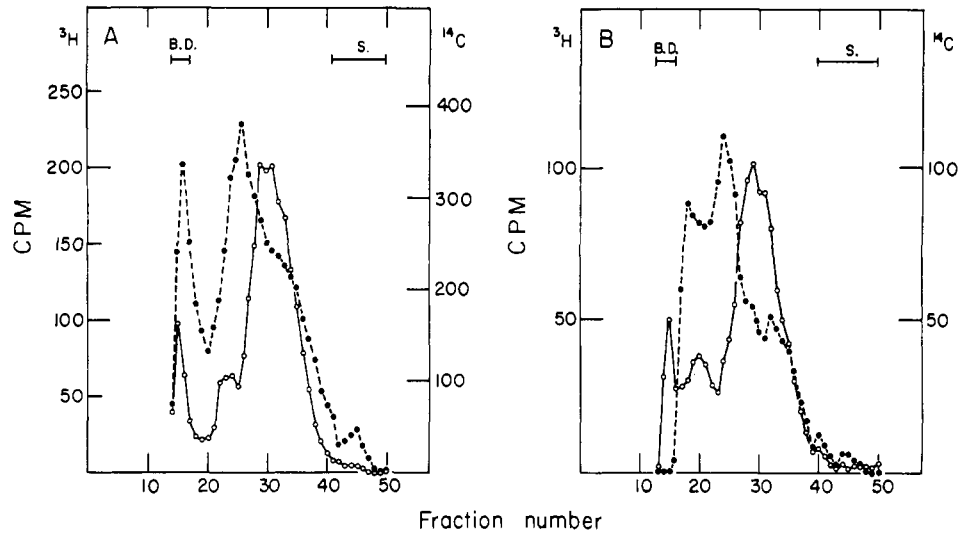


FIGURE 9: Relation of glycoprotein molecular weight to glycopeptide profiles. Glycoproteins of the membrane fraction from dividing and nondividing 3T3 cells labeled with either [^{3}H]glucosamine or [^{14}C]glucosamine were run separately on acrylamide gels. Peak M-2 glycoproteins of nondividing cells were combined with glycoproteins from fractions 26–30 (A) or 45–50 (B) of dividing cells. They were fractionated on Bio-Gel P-10 following exhaustive Pronase digestion. (●---●---●) [^{3}H]Glucosamine; (○---○---○) [^{14}C]glucosamine. B. D.: Blue dextran, S: sucrose.

of glycopeptides from fractions 45–50 there were found 3 major peaks. The molecular weight of these peaks were nominally 10,000, 6500, and 3100, respectively. A progressive increase in the glycopeptide size is seen as glycoproteins of increasingly lower molecular weights are examined. Larger glycopeptides seem to be preferentially associated with the smaller glycoproteins. The same results were obtained when glycoproteins were taken the reversed way. This preliminary analysis, in summary, suggests that there is great diversity in the glycopeptide of membrane protein, but that systematic changes in the size of these units is not a feature of virus transformation.

Discussion

Our purpose in these experiments has been to compare the glycoproteins and glycopeptides of normal and transformed cells, and to find an explanation for the quantitative and qualitative differences in carbohydrate compositions of normal and transformed cells reported by Wu *et al.* (1969) and by others (Buck *et al.*, 1970a; Ohta *et al.*, 1968). Wu *et al.* found decreased amounts of most neutral and amino sugars in transformed 3T3 cells as compared to normal cells; values were particularly depressed for sialic acids and *N*-acetylgalactosamine. Our principal conclusion from work reported here is that though we do not rule out subtle changes in linkages or composition of the sugar components of transformed cell glycoproteins, we cannot explain the observed carbohydrate differences seen by Wu *et al.* in terms of a gross change in the varieties of membrane glycoprotein present, or by a shortening of carbohydrate chains. We found almost identical profiles for the glycoproteins and glycopeptides of normal and transformed cells on polyacrylamide gels and Bio-Gel P-10 columns; we infer from this that no major shifts in the distribution of molecular weights of these molecules has occurred in transformed cells. Also there was no difference in the relative sialic acid content of glycopeptides from normal and transformed cells (Table III).

These findings were not restricted to the Swiss 3T3 mouse fibroblast lines described here. Normal and SV40 transformed lines of Balb/c 3T3 mouse fibroblasts, in which the transformed cells have only 35% of the sialic acid content of normal cells (Grimes, 1970), have very similar glycoprotein and glycopeptide profiles on polyacrylamide gels and on Bio-Gel P-10 columns. Moreover glycopeptide profiles of normal and transformed cells on DEAE-cellulose columns are identical (Sakiyama and Burge, 1972). DEAE-cellulose separations appear to be based on charge and thus glycopeptides of the same structure differing only in the absence of terminal sialic acid residue should be separated. Since they are not, these results are taken to support the model to be presented.

Before we suggest models that do account for the data of Wu *et al.*, it will be useful to summarize their findings more completely. They found overall decreases of 30–70% in the sialic acid, *N*-acetylglucosamine, and *N*-acetylgalactosamine contents of transformed Swiss 3T3 cells relative to normal Swiss 3T3 cells, whether expressed on a per cell basis or as microgram of carbohydrate per milligram of protein. Beyond this they found depressions of similar magnitude in the hexoses of transformed cells (fucose, mannose, and galactose). In all transformed cell fractions, sialic acid and *N*-acetylgalactosamine were more depressed than *N*-acetylglucosamine.

It is first important to point out that these shifts are of such magnitude (30–70%) that they cannot be explained by changes in glycolipids alone, which we estimate account for less than 20% of the carbohydrate of the 3T3 cell. Moreover, *N*-acetylglucosamine and mannose, which are not found in the glycolipids of 3T3 cells, are also depressed. We have not ourselves examined the glycolipids of normal and transformed 3T3 cells. A second class of carbohydrate-containing molecules, mucopolysaccharides, probably were an insignificant factor in the analysis of Wu *et al.* since these polymers are rapidly excreted and account for a very small part of the carbohydrate in the particulate fractions of cells (Hamerman *et al.*, 1965).

The results of the present work suggest that the carbohydrate depression seen in transformed cells must be explained as an absolute decrease in the number of glycoprotein mole-

cules per cell. As our results indicate that glycoproteins are for the most part membrane proteins, this depression may be explained either as (1) a decrease in the area of glycoprotein-containing transformed cell membranes or (2) a decrease in the density of glycoprotein per unit area of membrane. Both possibilities are compatible with our observation that glycoprotein profiles, glycopeptide profiles, and relative sialic acid content of glycopeptides are the same in normal and transformed cells. There may of course be more subtle changes in the transformed cell glycopeptides, as for instance, changes in linkage or sequence of sugars. At present, these remain undetected but will be the subject of future investigations.

Resolution of a Contradiction. Using pooled fractions of labeled glycopeptides from Sephadex G-50 columns, Meezan *et al.* measured the amounts of sialic acid relative to other amino sugars in normal and transformed cells. They found that normal 3T3 mitochondrial glycopeptides had relatively twice as much sialic acid as did SV40-3T3 mitochondria (Table I in Meezan *et al.*, 1969). We used in our experiments the same lines of 3T3 and SV40-3T3 cells as used by Wu *et al.*, but at roughly 15 passage numbers later; we found no difference in the sialic acid content of 3T3 and SV40-3T3 cell glycopeptide fractions (Figure 7, Table III). To be sure, different techniques were used and different glycopeptide fractions were examined; we used a bulk membrane glycopeptide fraction and identified sialic acid by elution position on Bio-Gel columns after mild hydrolysis, while Meezan and Wu used mitochondrial and nuclear glycopeptide fractions only, and identified and quantitated sugars through paper chromatography and isotope dilution methods. We believe that analytical procedures are defensible and measure roughly the same parameters. We can only suppose that the explanation of these experimental differences lies in an actual chemical difference occurring in the cells on extended passage. This kind of change is not without precedent. It has been noticed previously, for instance, that the sialic acid content of normal 3T3 cells decreases as these cells are continuously passaged (Grimes, 1970).

The important conclusion here, though, is that with two populations of cells having very different properties with respect to contact inhibition (see Figure 1) comparable glycopeptide fractions have the same sialic acid content (Figure 7, Table III). This result has also been obtained with a different set of normal and transformed cells, Balb/c 3T3 and SV40-3T3, (Sakiyama and Burge, 1972) and therefore we must conclude that the large decreases noted in the sialic acid contents of transformed cells are not due to "shortening" of glycopeptide chains, but are due more probably to an overall decrease in the number of glycoprotein molecule per milligram of cell protein. Apparently qualitative differences in the sialic acid content of glycopeptides, of the sort reported by Meezan *et al.*, can also occur in transformed cells, but these changes are not a necessary feature of a cell that has lost its ability to be contact inhibited. This question can be finally settled only in a situation where transformation occurs over a very short period of time in all cells of the culture, so that biochemical comparisons are not affected by genetic drift. We intend to approach this problem using a temperature-sensitive transforming mutant of Rous sarcoma virus.

A Multiplicity of Glycopeptide in Cell Surfaces. Since the role of glycopeptides of the cell surface glycoprotein is not understood, we did not know whether to anticipate a single "standard" glycopeptide attached to all membrane glycoproteins or a variety of glycopeptides, each covalently associated with a specific protein or set of proteins. Figures 8 and 9 indicate that fractionation of glycoproteins into specific molecular weight fractions prior to digestion by pronase results in a unique glycopeptide profile for each of the glycoprotein samples examined. Our results suggest that when structural studies are carried out many different glycopeptide structures will be found.

Differences between Normal and Transformed 3T3 Cells. Some subtle changes were found in the glycoproteins and glycopeptides of SV40 transformed 3T3 cells.

(1) A large (~150,000) glycoprotein present in the excreted fraction from normal 3T3 cells is missing in the excreted fraction of SV40 transformed cells (Figure 4).

(2) A large glycopeptide derived from a glycoprotein of ~60,000 is present in normal cells and absent in transformed cells (Figure 8C,D).

(3) The glycopeptides from the membrane fraction of virus-transformed cells all seem to have a high molecular weight shoulder which is not present in the homologous glycopeptide population of normal cell membranes (Figure 7). This alteration is not restricted to Swiss 3T3 cell transformed lines but is also found in SV40 transformed Balb/c 3T3 cells (Sakiyama and Burge, 1972). It may be related to a similar phenomenon described by Buck *et al.* in BHK 21 hamster cells transformed by Rous sarcoma virus (Buck *et al.*, 1970a,b). In our experiments, however, the effect was very minor, involving a difference of only a few fractions. We cannot guess at its significance.

The first two alterations noted above may be important, but are clouded by the difficulties in comparing two cell lines of unstable chromosome complement, which are separated by many cell generations. To ensure that the observed alterations are related to the transformation event and are not due to genetic drift which may occur in aneuploid cells, it will be necessary to examine several clones of independently transformed cells. We also intend to examine abortively transformed cells and cell-virus systems in which massive transformation can be achieved. It must also be kept in mind that 3T3 cell lines are not "normal" since they are aneuploid, but they are normal in the sense of being highly contact inhibited, in contrast to most tumor cells.

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Apparent Changes in Ribosome Conformation during Protein Synthesis. Centrifugation at High Speed Distorts Initiation, Pretranslocation, and Posttranslocation Complexes to a Different Extent†

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ABSTRACT: We prepared three intermediates in protein synthesis: *initiation complex* (f2 bacteriophage RNA·ribosome complex with formylmethionyl-tRNA$_f^{Met}$ (fMet-tRNA) in the P site). Part of the initiation complex was converted into a *pretranslocation complex* (f2 bacteriophage RNA·ribosome complex with fMet-Ala-tRNA in the A site and discharged tRNA in the P site). Part of the pretranslocation complex was converted into a *posttranslocation complex* (f2 bacteriophage RNA·ribosome complex with fMet-Ala-tRNA in the P site, having the A site vacant). We found that the pretranslocation complex sediments faster in a sucrose gradient centrifuged at high speed (230,000*g*, hydrostatic pressure 1200 atm) than either the posttranslocation complex or the initiation complex. The sedimentation patterns (including the per cent recovery in the peak fractions) of the pretranslocation and posttranslocation complexes centrifuged at high speed did not seem to be altered when the amount of the ribosome complexes in the gradients were decreased ninefold. In view of this it is unlikely that the difference in sedimentation velocities between the two complexes during high-speed centrifugation is due to a difference between them in reversible dissociation. In a gradient centrifuged at low speed (51,500*g*, hydrostatic pressure 300 atm) the pretranslocation and the posttranslocation complexes sedimented together. A mixture of pre- and posttranslocation complexes was first partially resolved by centrifugation through a sucrose gradient at high speed, then the fractions of the gradient containing either or both of the complexes were mixed and concentrated, and finally, the resulting solution was applied to a sucrose gradient which was centrifuged at low speed. In these conditions the two complexes appeared to sediment with an equal velocity. These and other data indicate that centrifugation at high speed does not result in an irreversible change in the sedimentation characteristics of the complexes, *i.e.*, probably does not cause an irreversible loss of components from them. All these findings may be explained by the following assumptions: the large hydrostatic pressure prevailing during centrifugation at high speed distorts the conformation of the two complexes to a different extent, and this in turn accounts for the difference between the sedimentation velocities of the two complexes. Consequently the difference in sedimentation velocities between the pre- and the posttranslocation complexes manifested at high-speed centrifugation is no proof for a difference in conformation between the complexes under physiological conditions.

Recently we developed an assay for measuring ribosome movement along the mRNA[1] during protein synthesis (Gupta *et al.*, 1971; see also Thach and Thach, 1971). These served to establish that (1) one step of the ribosome is as expected three nucleotides long, and (2) ribosome movement is triggered by the same factor (S_2) and GTP which triggers translocation of peptidyl-tRNA from site A to site P on the ribosome. The study required the preparation of three intermediates

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[1] Abbreviations used are: Init, initiation complex; Pre, pretranslocation complex; Post, posttranslocation complex; rcf, relative centrifugal force; GMPPCP, 5′-guanylylmethylenediphosphonate.