Comparative Studies on the Carbohydrate-Containing Membrane Components of Normal and Adenosine 3':5'-Cyclic Monophosphate-Treated Chinese-Hamster Ovary Cells

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1. We investigated some of the changes in plasma-membrane composition that accompany the alteration in cell growth and morphology induced by treating Chinese-hamster ovary cells with dibutyryladenosine 3':5'-cyclic monophosphate (dibutyryl cyclic AMP). 2. A double-labelling technique was employed in which normal cells were given ^{14}C -labelled precursor, and those treated with dibutyryl cyclic AMP were given ^3H label. L-Leucine, D-glucosamine, and L-fucose were used to label the membranes. 3. After 3 days growth, the two populations of cells were harvested by trypsin treatment, the cells were pooled, and plasma membranes isolated. Proteins and glycoproteins of the membranes were separated by electrophoresis on sodium dodecyl sulphate–polyacrylamide gels, and the radioactive profiles for ^{14}C and ^3H and the staining patterns with Amido Black were compared. 4. Although certain components of the membrane from treated cells showed marked quantitative changes, there was neither major addition nor major deletions of components. 5. Complete proteolysis of the mixed membranes, of the material released from the cell surface by trypsin, and of the glycoproteins released from the cells into the medium, gave a series of radioactive glycopeptides when either fucose or glucosamine was employed as precursor. 6. After such glycopeptides were fractionated on columns of Sephadex G-50, marked differences in the elution profiles of ^3H and ^{14}C were noted. Dibutyryl cyclic AMP evidently causes alterations in the overall composition of the carbohydrate components of the cell surface. It was not possible to decide whether this was solely the result of the same glycoproteins being formed but in different proportions, or the result of modifications of oligosaccharide side chains on some of the glycoproteins. 7. Some of the changes were not unlike the reverse of those that accompany the transformation of fibroblasts by oncogenic viruses, and our results lend credence to the idea that the lowered amount of cyclic AMP noted in transformed cells is responsible for their altered surface properties.

When Chinese-hamster ovary cells are treated with dibutyryladenosine cyclic 3':5'-monophosphate during culture *in vitro*, within a few hours they are converted from compact, randomly oriented cells that do not show strict contact inhibition of growth into elongated fibroblast-like cells which grow parallel to one another and form monolayers (Hsie *et al.*, 1971; Hsie & Puck, 1971). Further, whereas untreated cells form little or no collagen and are strongly agglutinated by wheat-germ agglutinin, the treated cells synthesize collagen and are only weakly agglutinated by plant lectins. Although these changes are only temporary and depend upon the continued presence of dibutyryl cyclic AMP in the medium, they do resemble the reverse of those that occur when fibroblast cells are transformed either by oncogenic viruses or by chemical mutagens. Moreover, it has been shown that when dibutyryl cyclic AMP is provided to transformed cells, their growth rate is also slowed and their morphological appearance reverts

temporarily to that of a normal fibroblast (Sheppard, 1971; Johnson *et al.*, 1971; Johnson & Pastan, 1972).

Cultured cells that have undergone transformation by virus show altered antigenic and chemical properties of their cell surface (Burger, 1971). In particular, there are marked changes in glycoprotein composition revealed in both isolated membranes and trypsin digests of the respective cell surfaces (Meezan *et al.*, 1969; Wu *et al.*, 1969; Buck *et al.*, 1970, 1971; Ohta *et al.*, 1968; Onodera & Sheinin, 1970). The altered agglutinability of Chinese-hamster ovary cells treated with cyclic AMP also suggests that surface carbohydrate groups have been modified or possibly masked in some way when they return to a fibroblastic state (Burger, 1971).

It was of interest, therefore, to investigate the composition of cell-surface components in Chinese-hamster ovary cells grown normally and those cultured in the presence of dibutyryl cyclic AMP, to see whether there were differences which resembled those noted

by other workers between normal fibroblasts and cells transformed by oncogenic virus. To accomplish this, we employed the double-labelling technique used by Wu *et al.* (1969), in which the two groups of cells to be compared are supplied with a radioactive precursor compound. However, while one group received the ^3H -labelled compound, the other was supplied with ^{14}C . At harvest the cells were combined and the relative contribution of ^3H or ^{14}C to various membrane components was investigated. In this study we used L-leucine as a precursor of polypeptide material, D-glucosamine to label amino sugar and neuraminic acid residues (Marshall & Neuberger, 1968), and L-fucose to label the L-fucosyl residues (Coffey *et al.*, 1964; Bekesi & Winzler, 1967) of glycoproteins and other complex carbohydrates.

Experimental

Methods

Cell cultures. The Chinese-hamster ovary cell line used was obtained from Dr. P. Cerutti and originated from clone CHO-KI (described by Kao & Puck, 1968). Cells were grown in roller bottles or in plastic bottles (Falcon, Cockeysville, Md., U.S.A.) on McCoy's 5A medium (McCoy *et al.*, 1959) with added 10% (v/v) foetal calf serum. In the labelling experiments, two roller bottles, each holding 100 ml of medium and with a surface area of 800cm^2 , were inoculated with 1×10^7 cells. One of the bottles contained 1 mM-6-N,2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate. Radioactive precursor was then added so that the roller bottle containing dibutyryl cyclic AMP received ^3H -labelled isotope and the untreated cells, ^{14}C . In certain experiments the labelling was reversed. The bottles were then rotated on a roller apparatus (Forma Scientific, Marietta, Ohio, U.S.A.) in an environmental chamber at 37°C for 72 h.

Radiochemicals

D-[1- ^{14}C]Glucosamine (51 mCi/mmol), D-[1- ^3H]glucosamine (2.6 Ci/mmol), L-[1- ^3H]fucose (0.92 Ci/mmol) and L-[1- ^{14}C]leucine (50 mCi/mmol) were obtained from Amersham-Searle, Des Plaines, Ill., U.S.A., L-[1- ^{14}C]fucose (45 mCi/mmol) from New England Nuclear Corp., Boston, Mass., U.S.A. and L-[4,5- ^3H]leucine (54 Ci/mmol) from Schwarz-Mann, Orangeburg, N.Y., U.S.A. The radiochemical purity of each compound, determined by paper chromatography followed by radiochromatogram scanning, was greater than 98%.

Trypsin treatment and harvest of cells. At the end of the growth period the medium was poured from each roller bottle and the cells adhering to the glass were washed three times with 100 ml of 0.16 M-NaCl at room temperature. The medium and washes from both roller bottles were combined and stored frozen

at -20°C until analysed. The cells were harvested by trypsin treatment. A trypsin solution (25 ml; 0.05%, w/v, in Hanks balanced salt soln.) was added to the bottles, which were then rolled gently. The trypsin solution was poured off and the bottles were rotated at 37°C to loosen the cells. After 5 min, the cells were suspended in 50 ml of 0.16 M-NaCl and recovered by centrifugation (100g; 5 min; 0°C). The supernatant fractions, which represent the trypsin digests, were combined, added to the original trypsin solution, and stored at -20°C .

Isolation of surface membranes. The surface membranes were isolated on sucrose density gradients as 'ghosts' by using a solution of fluorescein-mercuric acetate as a stabilizing reagent (Warren *et al.*, 1967; Warren & Glick, 1969). They were then dissolved in 0.04 M-Tris-glycine buffer, pH 7.8, containing 2% (w/v) sodium dodecyl sulphate, and boiled for 3 min.

Polyacrylamide-gel electrophoresis. The membrane protein solutions were dialysed against 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulphate and 0.1% mercaptoethanol for 3 h. Polyacrylamide gels (7.5%, w/v) containing 0.1% sodium dodecyl sulphate were prepared and run at pH 7.0, according to the procedure of Weber & Osborn (1969). For molecular-weight determinations, the following pure proteins were employed: trypsin, ovalbumin and bovine serum albumin.

Gels were stained with 0.2% Amido Black 10B for proteins or by periodic acid-Schiff reagent for carbohydrates (Zacharius *et al.*, 1969). For radioactivity determinations, an unstained gel was hardened by wrapping it in aluminium foil and placing it on solid CO_2 , and it was then sliced into 1 mm segments. Each slice was crushed and counted for radioactivity by liquid scintillation (see below). Approximately 80% of the measured radioactivity (both ^{14}C and ^3H) applied to the gels was consistently recovered by this procedure.

Pronase digestion. The solution of membrane proteins in sodium dodecyl sulphate was first diluted 10-fold with water and dialysed against 1 litre of 0.1 M-sodium phosphate buffer, pH 7.8, for 16 h. The trypsin digest was dried *in vacuo* and resuspended in the same phosphate buffer. An aqueous solution of CaCl_2 was added to both fractions to bring the Ca^{2+} concentration to 0.001 M and Pronase digestion was carried out at 37°C by the method of Spiro (1965).

Fractionation of the radioactive components in the medium. The medium was centrifuged at 8000g for 30 min to pellet whole cells and cellular debris. Trichloroacetic acid (40%, w/v) was then added to the clear supernatant fraction until its concentration was 7% (w/v). This preparation was kept at 3°C overnight and the precipitate collected by centrifugation. This pellet was washed three times with 7% (w/v) trichloroacetic acid, suspended in 0.1 M-sodium phosphate buffer, pH 7.8, and dialysed for 72 h against several

1 litre changes of the same buffer, and digested by Pronase according to the method of Spiro (1965).

The trichloroacetic acid-soluble fraction, which was considered to contain most of the acidic mucopolysaccharide (Kraemer, 1971*a,b*), was dialysed against water and saved for future studies. Hyaluronidase digestion of this material and other fractions was carried out with testicular hyaluronidase (Sigma Chemical Co., St. Louis, Mo., U.S.A.) at pH 5.3 for 24 h at 37°C. A large excess of enzyme was employed.

Gel filtration on Sephadex G-50. Glycopeptides released by Pronase digestion were subsequently chromatographed on Sephadex G-50. Two sizes of column were employed: a 55 cm × 1.5 cm column for the membrane digests in which there were only small quantities of material to analyse, and a 98 cm × 3.2 cm column for the larger amounts of material from the trypsin-treated material and medium. In the former case, 2 ml of material was loaded on to the column and 1.8 ml fractions were collected. In the latter, up to 5 ml was loaded on to the column and fractions collected were each 9.5 ml. Samples (0.5–1.0 ml) from each fraction were assayed for radioactivity. The columns were packed and equilibrated with 0.01 M-sodium phosphate buffer (pH 7.8) according to the recommendations of the manufacturer (Pharmacia, Piscataway, N.J., U.S.A.). The excluded volume was determined by using Blue Dextran; the included volume, by using sucrose. It should be noted that Phenol Red, which is frequently used as a coloured dye marker during gel chromatography, elutes well behind the 'sucrose' volume under our experimental conditions. We have also noted that free sialic acid (*N*-acetylneuraminic acid), which has approximately the same molecular weight as sucrose, eluted approximately two to three fractions ahead of sucrose on our larger Sephadex G-50 column. We assume that this molecule is sufficiently charged to be partially excluded from the gel due to ionic effects, whereas the Phenol Red may be non-specifically adsorbed.

Determination of radioactivity. The radioactivity of soluble samples diluted to 1 ml with water was counted in 10 ml of toluene-Triton X-100 (2:1, v/v) scintillant (Turner, 1968), by using a Packard Tri-Carb scintillation spectrometer. The channels were adjusted so that no counts from ^3H were found in the ^{14}C channel, and the extent of overlap of ^{14}C into the ^3H channel was set to 10%. Counting efficiency was 13.5% for ^3H and 43.5% for ^{14}C .

Other analytical techniques. Sialic acid was determined by the thiobarbituric acid method of Warren (1959), protein as described by Campbell & Sargent (1967) and sucrose as total carbohydrate by using α -naphthol (Dische, 1962). Cell numbers were estimated by means of a Coulter Counter. The amino acids released by Pronase were chromatographed overnight on Whatman no. 1 paper by using solvent (A): pyridine – ethyl acetate – water – acetic acid

(5:5:3:1, by vol.). The strips were scanned for radioactivity by using a Packard model 7201 radiochromatogram scanner, and then sprayed with ninhydrin to make visible the amino acid standards.

Results

Growth and morphology of the cells

The morphology of the cells was similar to that described by Hsie *et al.* (1971). Those grown in the absence of dibutyl cyclic AMP were compact and had only a few short cell processes. Continued growth led to the formation of multilayers. In the presence of dibutyl cyclic AMP, however, the cells had the appearance of typical fibroblasts. They were more obviously spindle-shaped, had longer and more numerous processes than their counterparts, and grew in parallel arrays. At confluency they showed pronounced contact inhibition of growth and no tendency to form multilayers. Cells grown in the presence of cyclic AMP (1 mM) also resembled fibroblasts, whereas sodium butyrate (2 mM) produced no marked change in cellular morphology.

In the labelling experiments, in which we used an inoculum size of 1×10^7 cells, we obtained a final cell count of 2×10^8 ($\pm 10\%$) in the case of the normal cells. This is equivalent to slightly over four cell generations. By contrast, the roller bottles containing dibutyl cyclic AMP gave approx. 40% fewer cells (1.2×10^8), indicating that the population doubling time had been decreased by about 25% in the presence of the drug.

At 72 h neither group had completely covered the surface of the glass, so that they were not confluent.

Incorporation of radioactive leucine, glucosamine and fucose into Chinese-hamster ovary cells

Table 1 summarizes the percentage distribution of the radioactivity supplied in the medium, in the trypsin digest and in the isolated membrane fractions from Chinese-hamster ovary cells that had been provided with radioactive leucine, glucosamine and fucose for 72 h in the presence or in the absence of dibutyl cyclic AMP. The ratios of these percentage values are also given.

Approximately ten times more ^3H label was provided than ^{14}C label; however, essentially similar results have been obtained when the labelling was reversed, thus precluding any isotope effects on the pattern of radioactive-label incorporation. Because there were approx. 40% more control cells than dibutyl cyclic AMP-treated cells at the time of harvest, relatively more ^{14}C than ^3H might have been expected to have been incorporated into each fraction. However, uptake by both sets of cells was comparable.

Table 1. Percentage distribution of radioactivity in various labelled fractions isolated from Chinese-hamster ovary cells supplied with labelled leucine, glucosamine and fucose in the presence and in the absence of dibutyryl adenosine 3':5'-cyclic monophosphate

	Leucine			Glucosamine			Fucose		
	^3H	^{14}C	$^{14}\text{C}/^3\text{H}$	^3H	^{14}C	$^{14}\text{C}/^3\text{H}$	^3H	^{14}C	$^{14}\text{C}/^3\text{H}$
Radioactivity introduced (μCi)	50.00	5.00		130.00	11.00		125.00	10.00	
Radioactivity recovered from media (%)									
Total	55.00	50.00		75.00	83.50		94.20	95.50	
Trichloroacetic acid-insoluble	2.72	2.60	0.95	2.58	2.27	0.88	1.30	1.30	1.00
Trichloroacetic acid-soluble	0.56	0.40	0.71	1.31	1.09	0.83	0.17	0.30	1.78
Radioactivity lost during dialysis	51.72	47.00		71.11	80.14		92.73	93.90	
Radioactivity recovered from cell surface (%)									
Trypsin-treated fraction	0.96	2.00	2.08	0.87	0.64	0.73	0.16	0.17	1.03
Membrane fraction	1.26	2.20	1.74	0.11	0.27	2.38	0.15	0.16	1.07

Leucine. In the experiments with leucine, only about one-half the radioactivity supplied was recovered in the medium at the time of harvest. About 95% of this was diffusible upon dialysis. However, both sets of cells had released some radioactive substances of high molecular weight into the medium that were retained in the dialysis sac. Most of this was precipitated upon addition of trichloroacetic acid and had a $^{14}\text{C}/^3\text{H}$ ratio close to 1. Trypsin treatment of the cells, on the other hand, released comparatively more ^{14}C than ^3H .

Similar results were obtained when the $^{14}\text{C}/^3\text{H}$ ratios of the pooled membrane fractions were measured. It would appear that leucine incorporation into surface proteins was about twice as high in the normal cells as in those treated with dibutyryl cyclic AMP. In both types of cells, the isolated membranes contained slightly more radioactivity than did the materials released from the cell surface by trypsin.

Glucosamine. When glucosamine was supplied to the cells about 75% of the ^3H and 83% of the ^{14}C was recovered in the medium. As with leucine, some of this radioactivity was in molecules of high molecular weight, some of which could be precipitated with trichloroacetic acid. The ratio $^{14}\text{C}/^3\text{H}$ in both the trichloroacetic acid-insoluble and trichloroacetic acid-soluble fractions was less than 1. This suggests that the cells treated with dibutyryl cyclic AMP secreted more macromolecular product into the medium than did the normal cells. Goggins *et al.* (1972) have also noted that Sendai virus 40 (SV 40)-transformed 3T3 cells secrete increased amounts of sulphated acidic mucopolysaccharides into the medium after they are given dibutyryl cyclic AMP.

Trypsin solubilized a large proportion of the radioactivity associated with cell surface. Thus, in the case of the cells treated with dibutyryl cyclic AMP, about eight times as much radioactive label was recovered in the trypsin-treated material as in the cell membranes. In untreated cells the distribution of ^{14}C between trypsin-treated material and membrane was about 3:1. Clearly, the availability of surface glycopeptides susceptible to release by trypsin increased when cells were grown with dibutyryl cyclic AMP in the medium. Further, even if it is assumed that the recovery of plasma membranes is only partially complete, it would appear that trypsin releases a large percentage of the surface carbohydrates that contain amino sugars from both sets of cells.

The $^{14}\text{C}/^3\text{H}$ ratio of the combined trypsin-treated material was slightly below 1, again indicating a preferential incorporation of glucosamine into this fraction by the cells treated with dibutyryl cyclic AMP. Others have also reported that fibroblasts have greater amounts of sialic acid and amino sugars associated with their surfaces than have their transformed counterparts (Meezan *et al.*, 1969; Ohta *et al.*, 1968; Grimes, 1970). By contrast, however, the mem-

EXPLANATION OF PLATE I

Staining pattern with Amido Black of the plasma-membrane fraction from Chinese hamster ovary cells

Components were separated by electrophoresis on polyacrylamide gels containing sodium dodecyl sulphate. The gel shown is from an experiment with a mixed population of cells ($\pm$ dibutyryl cyclic AMP). The molecular weights of the various stained bands (I–XXII) are only approximate. Migration is downwards to the anode.

branes gave a $^{14}\text{C}/^3\text{H}$ ratio of 2.38, although it has to be remembered that the number of normal cells was greater.

Fucose. Fucose was utilized very poorly by both sets of cells and more than 95% of the total radioactivity remained in the medium at the end of the growth period. Only a small portion of this was in compounds of high molecular weight and most of these were precipitated by trichloroacetic acid. However, of all the fractions analysed, only the trichloroacetic acid-soluble material showed a marked divergence of its $^{14}\text{C}/^3\text{H}$ ratio from 1. With regard to cell-surface material, the radioactive fucose, unlike the label from glucosamine, was distributed about equally between the plasma-membrane fraction and the trypsin-treated material.

Disc gel electrophoresis of isolated membrane solubilized in sodium dodecyl sulphate

Isolated membranes double-labelled with ^3H and ^{14}C were dissolved in sodium dodecyl sulphate solution at 100°C and the membrane components separated by electrophoresis on sodium dodecyl sulphate-polyacrylamide gels. The pattern of staining by Amido Black shown in Plate 1 was consistently reproduced in each experiment, although there were slight variations in the total distance migrated. In addition, in a series of separate experiments run without mixing, we isolated membranes from normal Chinese-hamster ovary cells and from Chinese-hamster ovary cells grown in the presence of dibutyryl cyclic AMP and subjected these membranes to gel electrophoresis. Again, the staining pattern appeared similar to that in Plate 1, and we could not detect any major qualitative differences between the two sets of membranes. Increasing the concentration of sodium dodecyl sulphate and mercaptoethanol in the solubilizing buffer or increasing the amount of sodium dodecyl sulphate in the gels did not alter this pattern.

A total of 20–22 stained bands with apparent molecular weights ranging from about 160000 to 12000 can be seen. It is possible that the densely staining, rapidly migrating bands represent breakdown fragments of larger molecules. They have been noted, however, in each of more than 20 experiments; and we have taken great care to avoid bacterial contamination or proteolysis of the isolated membranes before electrophoresis. Unlike the erythrocyte (Winzler, 1969; Bretscher, 1971), there was no single, major component that stained deeply with periodic acid-Schiff reagent under the experimental conditions used, although there were several minor bands located throughout the gel.

The distribution of radioactivity along the gels is shown in Fig. 1 for the three sets of labelling experiments. Note that the scale for ^3H (right) is ten times

that for ^{14}C (left). This is close to the ratio of $^{14}\text{C}/^3\text{H}$ supplied to the cells. Any deviation from this ratio can be seen clearly wherever the ^3H and ^{14}C lines are offset from each other.

With leucine the membranes had a $^{14}\text{C}/^3\text{H}$ ratio greater than 1 (Table 1), indicating a greater contribution of leucine radioactivity to the membranes of normal cells. This is also reflected in the gel pattern (Fig. 1a), which shows an enhancement with regard to ^{14}C in each region and is probably due, at least in part, to the greater number of normal cells that were present at the start of the isolation. Few, if any, new bands uniquely labelled with either isotope can be discerned. However, the ratio $^{14}\text{C}/^3\text{H}$ clearly does not remain constant along the gel, and it would appear that the same components may be present in both sets of membranes but in relatively different amounts. The staining by Amido Black shown in Plate 1 is evidently non-stoichiometric, as the densely staining bands of low molecular weight did not incorporate proportionately higher amounts of radioactivity than several more lightly staining regions further up the gel. The amount of radioactivity present at the top of the gel is also very low, suggesting that the membrane polypeptides were completely dissociated in the sodium dodecyl sulphate solution.

Our present results suggest that the quality of membrane-associated macromolecules which become labelled with leucine are not influenced greatly by the presence or the absence of dibutyryl cyclic AMP in the medium. The relative proportions of the various components, however, do appear to change in the presence of dibutyryl cyclic AMP.

The radioactive gel patterns of membranes labelled with glucosamine are shown in Fig. 1(b). Glycoproteins were found throughout the gel and there was a fairly close coincidence for ^{14}C and ^3H , except in a region close to the origin and in slices 58 and 65, where the profiles diverged markedly. Each of these peaks was enriched with ^{14}C . The material close to the top of the gel may be mucopolysaccharides, which penetrate such gels very poorly, owing to their high molecular weights and lack of affinity for sodium dodecyl sulphate.

The material in slice 58 ran close to the major protein band XXI, but we have no evidence that it is part of that component. The fast-running label in slice 65 usually seemed to migrate into an unstained region of the gel slightly ahead of protein band XXII. It may be a glycolipid; these are known to move rapidly towards the anode in sodium dodecyl sulphate-polyacrylamide gels and are usually found close to the region of the Bromophenol Blue dye marker. The gel profiles again suggest that the change in cell morphology induced by dibutyryl cyclic AMP is not accompanied by the appearance of new membrane components. Certain components, however, do appear to be synthesized in different amounts.

Fig. 1. Incorporation profiles of ^3H (○) and ^{14}C (●) radioactivity into plasma-membrane fractions from Chinese-hamster ovary cells

(a) Incorporation of L-leucine; (b) incorporation of D-glucosamine; (c) incorporation of L-fucose. Labelled components were separated by electrophoresis and the radioactivity was determined in 1 mm slices. Migration is to the right. For further details see the text.

Except for a region at the top of the gels (Fig. 1c), the membranes labelled with fucose showed fairly constant proportions of ^{14}C and ^3H in each radioactive band, as predicted by the $^{14}\text{C}/^3\text{H}$ ratio associated with the total membrane fraction (see Table 1). Glycoproteins were again found throughout the gel, but, owing to the slight differences in migration rates that occur from run to run, it is difficult to compare in detail the radioactive profile seen in Fig. 1(c) with those noted with glucosamine or leucine in 1(a) and 1(b). However, it is clear that the fast-moving components that became labelled with glucosamine did not incorporate appreciable amounts of radioactive fucose.

Sephadex G-50 gel filtration of Pronase-digested membranes

Portions of the double-labelled membrane fractions were exhaustively digested with Pronase and glycopeptide components separated by size on a column (55 cm $\times$ 1.5 cm) of Sephadex G-50. The smaller G-50 column (with a void volume of about 28 ml) was used in these experiments because the total amounts of radioactivity and material were low. Only the results with radioactive glucosamine and fucose are reported.

Fig. 2(a) shows the elution profile of ^3H and ^{14}C for cells labelled with glucosamine. A large proportion of the ^{14}C , but not of the ^3H , was eluted at the void front. When this material was treated with hyaluronidase and then rechromatographed, more than 70% of the radioactivity was eluted in the excluded volume (peak I) of the column. This indicates that it was probably acidic mucopolysaccharide, and most likely corresponds with the material that did not penetrate the gel during electrophoresis (see Fig. 1b). This observation again suggests that the untreated cells had more mucopolysaccharide associated with their membranes than did the cells grown in the presence of dibutyryl cyclic AMP. The later peaks are probably glycopeptides resulting from the proteolysis of membrane glycoproteins. The partially included peaks II and III had similar proportions of ^3H and ^{14}C , whereas the included peak IV was considerably enriched with ^{14}C compared with ^3H .

The radioactive profile observed after labelling with fucose (Fig. 2b) did not show the large peak at the void front, and the elution patterns for ^3H and ^{14}C were qualitatively similar. However, the material of low molecular weight eluted between fractions 42 and 52 was markedly enriched with ^3H , which is the reverse of the situation observed with glucosamine label (Fig. 3a). In the presence of dibutyryl cyclic AMP, cells evidently incorporate more fucose but less glucosamine into short-chain oligosaccharide components of glycoproteins.

Fig. 2. Comparison of the glycopeptides released from plasma membranes by Pronase chromatographed on a column (1.5 cm $\times$ 55 cm) of Sephadex G-50

The elution of ^3H ($\circ$) and ^{14}C ($\bullet$) radioactivity is shown; each fraction was 1.8 ml. (a) shows the pattern with D-glucosamine, (b) with L-fucose. For details of the peaks I-IV see the text.

Sephadex G-50 gel filtration of the trypsin-treated material

The fraction released from the cells by trypsin was treated with Pronase and portions were chromatographed on a large column (98 cm $\times$ 3.2 cm) of Sephadex G-50. Fig. 3 shows the elution profile for the three types of double-label experiment.

Fig. 3(a) indicates that the digestion with Pronase was complete. All of the radioactivity from leucine was eluted as a narrow symmetrical band between

Fig. 3. Comparison of the glycopeptides released from the trypsin-treated cell material by digestion with Pronase

Radioactive material was chromatographed on a column (3.2 cm $\times$ 98 cm) of Sephadex G-50. The elution of ^3H (○) and ^{14}C (●) radioactivity is shown; each fraction was 9.5 ml. (a) shows the pattern after L-leucine labelling, (b) after D-glucosamine labelling and (c) after L-fucose labelling.

fractions 70 and 80, which coincided with the region of the 'sucrose' volume. When this radioactive material was concentrated and chromatographed on paper by using solvent (A), all of the detectable radioactivity was associated with L-leucine, and not with short-chain peptide fragments.

The profile for glucosamine label was very complex and the digest was clearly heterodisperse (Fig. 3b). Further, the surface materials from cells grown in the presence of dibutyryl cyclic AMP displayed an elution pattern distinctly different from that of the untreated cells. Some double-labelled materials ($^{14}\text{C}/^3\text{H}$ ratio, 0.055) were eluted at the void-volume front and had, therefore, a molecular weight of 30000 or greater. More than 80% of these were susceptible to a 24 h digestion by hyaluronidase. The fibroblast-like cells appeared, therefore, to have more acidic mucopolysaccharide, which can be released by trypsin, than did their normal counterparts. As the elution continued, ^{14}C and ^3H profiles continued to diverge. For example, the average $^{14}\text{C}/^3\text{H}$ ratio between fractions 40 and 50 was approx. 0.06, whereas between 50 and 60 it had fallen to about 0.04. A large portion of the ^{14}C and ^3H radioactivity was eluted as a broad asymmetric peak between fractions 68 and 80, with a $^{14}\text{C}/^3\text{H}$ ratio of 0.1. It is within this region that oligosaccharides of molecular weight less than 1500 eluted. In one experiment we noted that the ^3H -labelled peak appeared slightly ahead of this region; its peak was about two fractions in advance of the ^{14}C -labelled peak, and was presumably in materials of slightly higher molecular weight or of a more acidic nature (see the Experimental section). We believe that this was not an artifact (the trypsin-treated materials were mixed before Pronase digestion), but was due to better chromatographic resolution in this particular run. The band of ^3H did, nevertheless, show considerable overlap with the ^{14}C and tailed into the region of the 'sucrose' volume. No fractions were collected that contained ^3H or ^{14}C exclusively.

The elution profiles of the radioactive glycopeptides that were labelled by fucose are shown in Fig. 3(c). As with the Pronase-digested membranes (see Fig. 2b), only very small amounts of radioactivity were detected at the void-volume front, but broad asymmetric bands were eluted between fractions 40 and 60 and between fractions 70 and 80. Again, the elution profile of the glycopeptides from the treated cells was distinct from that of the control cells. The most apparent differences are in fractions 40–50, which were enriched with respect to ^{14}C , and in fractions 50–60 and 70–80, which had low $^{14}\text{C}/^3\text{H}$ ratios.

Sephadex G-50 gel filtration of the trichloroacetic acid-insoluble fraction

Membrane turnover results in the release of component glycoproteins into the medium (Hughes *et*

Fig. 4. Comparison of the glycopeptides released from the glycoproteins in the medium by digestion with Pronase

Radioactive material was chromatographed on a column (3.2cm × 98cm) of Sephadex G-50. The elution of ^3H (○) and ^{14}C (●) radioactivity is shown; each fraction was 9.5ml. (a) shows the pattern after L-leucine labelling, (b) after D-glucosamine labelling and (c) after L-fucose labelling. The glycoproteins were regarded as material precipitated from the medium by trichloroacetic acid.

products secreted by the cell plus components released from the membrane, but large quantities of serum proteins, which are standard components of the medium, were also present. Nevertheless, the digestion with Pronase appeared to be complete, as the radioactivity introduced by labelled leucine was all eluted in the included volume of the column (Fig. 4a). The radioactive profile of the trichloroacetic acid digest from cells supplied with glucosamine (Fig. 4b) shows three major regions of radioactivity: a completely excluded peak at the void-volume front, rich in ^3H ; a broad, presumably heterodisperse, peak, which contains the bulk of the digested material and consists of partially included glycopeptides; and, lastly, a broad peak eluted between fractions 65 and 80. As the included volume (measured in terms of sucrose elution) occurs within this region, glycopeptides of molecular weight 1500 or less are again present. This peak was comparatively richer in ^{14}C and ^3H , whereas the partially included peak contained more material from the dibutyl cyclic AMP-treated cells.

The profile obtained with radioactive fucose (Fig. 4c) was not unlike that with glucosamine. Again, however, fucose did not contribute greatly to the material eluted at the void-volume front, and the peak in the region of the included volume showed a low, rather than high, $^{14}\text{C}/^3\text{H}$ ratio.

Discussion

Although changes are believed to occur in surface carbohydrate materials after cells become transformed by oncogenic virus or by chemical mutagens, the comparisons that have been made may not always have been valid because such cells are probably subject to considerable genetic drift during continued subculture, which might be responsible for the observed differences in composition. In addition, results have not always been reproduced consistently in other laboratories. Sakiyama & Burge (1972), for example, have argued that, although there may be demonstrable differences in overall carbohydrate content, transformed cells having substantially less carbohydrate than normal cells, the glycopeptides and glycoproteins of the membranes of the two cell types appear indistinguishable by either polyacrylamide-gel electrophoresis or gel chromatography. Warren *et al.* (1972) have also been unable to detect the major changes in membrane glycolipid composition that have been reported by others to accompany transformation (Rapport *et al.*, 1959; Hakomori & Murakami, 1968; Mora *et al.*, 1969), although their experiments did show differences in the content of glycopeptides released by trypsin.

In the studies described here, we have compared the glycoproteins and glycopeptides of the surface of Chinese-hamster ovary cells with those from similar

al., 1972). The material that was isolated from the dialysed medium by precipitation with trichloroacetic acid was hydrolysed, therefore, by using Pronase and chromatographed on the large column of Sephadex G-50. Not only was this fraction likely to contain

cells treated with dibutyl cyclic AMP. In each experiment both populations originated from the same culture bottle, and only three to four cell generations had occurred before harvest. Moreover, the morphological changes induced by the drug appear within a few hours of treatment and are reversed as soon as it is removed. It is most unlikely therefore that the changes we observed were the result of genetic differences between the two groups of cells, and it seems reasonable to suspect that they are the direct consequence of increased amounts of dibutyl cyclic AMP in the cells. It is recognized, of course, that Chinese-hamster ovary cells are not tumorigenic. However, as outlined in the introduction, they do show many of the characteristics of the typically transformed cell line, whereas their counterparts treated with dibutyl cyclic AMP exhibit a more 'normal' type of fibroblastic growth.

Both sets of membranes appeared to contain a similar group of protein components, as judged by their staining patterns on sodium dodecyl sulphate-polyacrylamide gels. Further, the radioactive banding patterns with leucine were qualitatively alike, although it was again clear that major quantitative changes had occurred in a number of components. Similarly, with L-fucose and D-glucosamine, no region of the gel appeared to be uniquely labelled with either isotope, but the radioactive profiles often showed a marked lack of coincidence. From these observations it seems reasonable to suppose that neither set of membranes contained a major protein or glycoprotein species that distinguished it from the other, although a minor change cannot be completely ruled out. It is realized, of course, that a minor shortening or lengthening or even a deletion or addition of a carbohydrate chain on a glycoprotein need not alter the molecular weight of such a component sufficiently to change its migration on sodium dodecyl sulphate-polyacrylamide gels. The relative differences in the ^3H or ^{14}C profiles noted with glucosamine or with fucose could therefore be due to two causes, or a combination of these two events. Either the same glycoproteins are formed after dibutyl cyclic AMP treatment but in different proportions, or the same polypeptides are formed but they carry different or modified oligosaccharide chains. The latter alternative could be consistent with the theory that carbohydrate chains on tumour cells are 'incomplete' (Hakomori & Murakami, 1968).

These alternatives, however, are not easy to distinguish by subjecting the glycopeptides of either the isolated membranes or the trypsin-treated material to gel filtration because the compounds were so varied with respect to molecular size. In this respect, our results merely confirmed that significant changes in glycopeptide composition accompanied the alteration in cell morphology induced by dibutyl cyclic AMP. However, it is noteworthy that in the experi-

ments with fucose, the trypsin-treated material of the normal cells was richer in a population of more rapidly migrating glycopeptides (in terms of Sephadex G-50 elution) than those treated with dibutyl cyclic AMP, and the results were similar to those of Buck *et al.* (1970, 1971) when they compared virus-transformed cells with normal fibroblasts. Our results support the idea that one of the early consequences of transformation is a fall in the amount of cyclic AMP in the cell (Otten *et al.*, 1971, 1972); this event in turn may be responsible for the altered morphological and surface properties of the transformed cell.

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