Growth and Metabolism of Fucosylated Plasma-Membrane Glycoproteins in Mouse Neuroblastoma N2a Cells

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The presence of 1.0 mM-dibutyryl cyclic AMP ($N^6, O^{2'}$ -dibutyryl adenosine 3':5'-cyclic monophosphate) and 1.5 mM-theophylline completely inhibits the growth of mouse neuroblastoma N2a cells by 24–36 h. When compared with N2a cultures without inhibitors (controls), the proportion of cells in S phase, measured by radioautography with [^3H]-thymidine, was decreased from 55 to 12%. In addition, the presence of the inhibitors decreased apparent [^3H]fucose incorporation into glycoproteins by 50%, and removing the inhibitors resulted in a rapid recovery of both DNA synthesis and glycoprotein metabolism. Measurement of intracellular acid-soluble radioactive fucose revealed that decreased fucose uptake could account for the apparent change in incorporation. Removing dibutyryl cyclic AMP and theophylline from the medium resulted in a rapid uptake of radioactive fucose to within control values, which illustrated that the inhibitors decreased transport of the carbohydrate, although the cells remained viable. Treatment with dibutyryl cyclic AMP and theophylline also reversibly inhibited glycoprotein degradation. Plasma membranes isolated from growing cells and from growth-inhibited cells labelled with [^{14}C]fucose and [^3H]fucose respectively were co-electrophoresed on sodium dodecyl sulphate/polyacrylamide gels. These displayed no apparent differences in synthesis of specific membrane glycoproteins. Electrophoresis of plasma membranes isolated from cultures pulse-chased with [^{14}C]fucose and [^3H]fucose was used to discern turnover patterns of specific plasma-membrane glycoproteins. High-molecular-weight glycoproteins exhibited rapid rates of turnover in membranes from growing cells, but moderate turnover rates in growth-inhibited cells and cells reversed from growth inhibition. These data indicate that growth arrest of N2a cells results in alterations in the metabolic turnover of plasma-membrane glycoproteins.

Cell biologists have been greatly interested in the relationship of eukaryotic plasma-membrane structure and metabolism to cell growth, especially with respect to the differences between normal and transformed cells. Treating cell surfaces with proteolytic enzymes stimulates growth of contact-inhibited cells (Burger, 1970), and proteinase inhibitors inhibit growth of cells in culture (Chou *et al.*, 1974; Collard & Smets, 1974). Lectin binding and cell agglutination are increased after transformation (Burger, 1971; Ozanne & Sambrook, 1971; Barbarese *et al.*, 1973; Shoham & Sachs, 1974) and during mitosis of untransformed cells (Shoham & Sachs, 1974). Uptake of phosphate and hexoses is increased in transformed and decreased in contact-inhibited 3T3 cells (Cunningham *et al.*, 1975). The appearance and disappearance of specific glycoproteins during the cell cycle or as a result of viral transformation has been well documented (Warren *et al.*, 1973; Glick *et al.*, 1974; Gahmberg & Hakomori, 1974; Hynes & Bye,

1974; Shin *et al.*, 1975), especially with respect to a membrane glycoprotein of mol.wt. 200 000–250 000, which is present in largest quantity during the G1 phase of the cell cycle, but disappears during M phase or viral transformation (Gahmberg & Hakomori, 1974; Hynes & Bye, 1974).

Several studies have indicated that plasma membranes are dynamic structures, such that their macromolecular components are constantly synthesized and degraded and that heterogeneous rates can be observed for the turnover of specific components (Dehlinger & Schimke, 1971; Kaplan & Moskowitz, 1975; Landry & Marceau, 1975; Tweto & Doyle, 1975; Mathews *et al.*, 1976; Hudson & Johnson, 1977b). Because metabolism of the plasma membrane can reflect the growth state of the cell, particularly with respect to the cell cycle or transformation-dependent glycoproteins, one might expect that turnover rates for these macromolecules would differ as a function of growth. A correlation

between growth and membrane glycoprotein metabolism is suggested on the basis of studies that showed that both incorporation of carbohydrates into membrane glycoproteins (Bosmann & Winston, 1970; Gerner *et al.*, 1970; Nowakowski *et al.*, 1972; Graham, 1973), degradation (Rieber *et al.*, 1975) and turnover of specific glycoproteins (Kaplan & Moskowitz, 1975) varied with phase of the cell cycle or transformation.

Previous work in our laboratory has demonstrated that mouse neuroblastoma N2a cells undergo heterogeneous rates of plasma-membrane glycoprotein turnover. Treating this cell line with dibutyryl cyclic AMP (N^6, O^2' -dibutyryladenosine 3':5'-cyclic monophosphate), which has been shown to result in growth arrest of neuroblastoma cells (Prasad *et al.*, 1973; Prasad & Sheppard, 1972), as well as other cell types (Sheppard, 1971; Froehlich & Rachmeler, 1972; Frank, 1972; Greengard, 1978), leads to alterations in species of glycoproteins released from the cell surface (Truding *et al.*, 1975). We have been interested in the effect of changes in growth state on the turnover of plasma-membrane glycoproteins of neuroblastoma N2a cells.

Materials and Methods

Materials

Radioactive chemicals were purchased from New England Nuclear (Boston, MA, U.S.A.); cell culture-medium components and antibiotics were from Grand Island Biological Co. (Grand Island, NY, U.S.A.). Non-radioactive fucose, dibutyryl cyclic AMP (sodium salt) and theophylline were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); NTB2 nuclear track emulsion, Dektol and fixer were from Eastman Kodak Co. (Rochester, NY, U.S.A.). The [^3H]fucose (sp. radioactivity 12.1 Ci/mmol) was diluted with unlabelled L-fucose and used at 1–3 $\mu\text{Ci/ml}$ (2.6–2.8 nmol/ml); [^{14}C]fucose (sp. radioactivity 53.2 $\mu\text{Ci/mmol}$) was used undiluted at 0.25 $\mu\text{Ci/ml}$ (4.7 nmol/ml); and [^3H]thymidine (sp. radioactivity 52.8 Ci/mmol) was used diluted with unlabelled thymidine at 2 $\mu\text{Ci/ml}$ (0.11 nmol/ml) of complete media.

Neuroblastoma-cell growth and growth inhibition

The N2a line of mouse neuroblastoma cells (obtained from Dr. A. Telser, Northwestern University Medical School) were maintained as monolayer cultures by the method of Hudson & Johnson (1977b). Subconfluent monolayer cultures were grown for 2 days after the last transfer to allow time for their recovery from Viokase treatment before they were incubated with radioactive fucose or growth inhibitors. Growth inhibition was accomplished by incubating cells in complete medium containing 1 mM-dibutyryl cyclic AMP and 1.5 mM-

theophylline for 24–36 h. To produce reversal of growth inhibition, the inhibitors were removed and the cells were reincubated in complete medium. The rate of cell growth was monitored either by directly counting cells in a haemocytometer or by measuring total cell protein (Lowry *et al.*, 1951) with bovine serum albumin as standard.

Incubation with radioactive precursors

Growing or growth-inhibited cultures were labelled with either [^3H]- or [^{14}C]-fucose in complete medium with or without growth inhibitors. Medium containing [^3H]fucose was supplemented with unlabelled 2.5 μM -L-fucose to avoid the possibility that fucose was rate limiting to cell metabolism (Hudson & Johnson, 1977a).

Fucose incorporation into whole cells

Neuroblastoma cells, grown in plastic tissue-culture dishes (60 mm diameter) and labelled with [^3H]fucose as indicated, were washed twice with ice-cold 0.15 M-NaCl; 0.1 ml of 1 M-NaOH plus 0.9 ml of 0.15 M-NaCl was then added to each plate and the viscous lysate was removed into a thick-walled test tube containing 1 ml of ice-cold 10% (w/v) trichloroacetic acid. The culture plate was washed with 1 ml of ice-cold 5% trichloroacetic acid to remove residual cell material, and the wash was added to the original lysate. Acid-insoluble material was pelleted by centrifugation at 2000g for 10 min; the supernatant was kept and the pellet washed with 5% trichloroacetic acid. The second supernatant fluid was pooled with the first and, where indicated, was assayed for acid-soluble radioactivity. The pellet was resolubilized in 0.5 ml of 1 M-NaOH, a 0.2 ml sample was removed for protein determination by the method of Lowry *et al.* (1951), and 0.06 ml of 5 M-acetic acid was added to the remaining acid-insoluble material to neutralize the pH. A portion (0.2 ml) was assayed for radioactivity in 3a70b scintillation fluid (Research Products International Corp., Elk Grove Village, IL, U.S.A.).

Radioautography

Growing, growth-inhibited, or growth-inhibited and then reversed cell monolayers (which had been cultured on coverslips), were incubated with 2 μCi of [^3H]thymidine/ml for 30 min. The coverslips were washed 5 times with 2 ml of complete medium and then placed in Carnoy's solution (4 parts of ethanol to 1 part of acetic acid) for 2 h at -20°C . The coverslips were subsequently dipped for 2 min successively in 70%, 95% and absolute ethanol, allowed to dry, and then mounted on microscope slides.

The following procedure was then carried out in a darkroom illuminated with a sodium-vapour light. Kodak NTB2 nuclear track emulsion was melted by heating in a 37°C water bath for 1 h. The slides were

dipped in the emulsion, dried under circulating air for 1 h, and then stored refrigerated (4°C) in a light-proof box containing Drierite (Fisher Scientific Co., Pittsburg, PA, U.S.A.) for 2 weeks. The slides were developed by dipping them for 2 min in Dektol and then for 3 min in fixer. After washing under running water for 30 min, the cells were stained with Mallory's stain and then dried by successive immersion in 70%, 95% and absolute ethanol. Before mounting with a second coverslip, the cells were coated with Permount (Fisher Scientific Co.); labelled and unlabelled nuclei were counted for radioactivity to determine percentages of the cells undergoing DNA synthesis (S-phase).

Plasma-membrane isolation and gel electrophoresis

Neuroblastoma-cell plasma membranes were isolated by a modification of the Zn^{2+} method of Warren *et al.* (1966) as described by Mathews *et al.* (1976), except that, after centrifugation of the cell homogenate over a discontinuous 65%/40% sucrose gradient, the membrane fraction was removed from the 65%/40% interface and centrifuged for 20 min at 3400g. This membrane pellet was resuspended in 0.15 M-NaCl and frozen at -80°C.

Plasma-membrane glycoproteins were separated on sodium dodecyl sulphate/urea/polyacrylamide gels essentially as described by Fairbanks *et al.* (1971). Profiles of neuroblastoma plasma-membrane proteins were determined by staining the gels with Coomassie Blue, and peaks corresponding to protein were scanned as described by Mathews *et al.* (1976). Approximate molecular weights were estimated by comparing electrophoretic mobilities of membrane proteins and glycoproteins with those of proteins of known molecular weight. Bovine serum albumin (68000), egg albumin (45000), chymotrypsin (25000) and cytochrome *c* (13500) were used as standards.

Treatment of radioactive gels

Gels containing radioactively labelled membrane components were frozen in solid CO_2 /ethanol and sliced into 1.0 mm fractions with a Bio-Rad Gel Divider (model 190). Each slice was shaken overnight in a scintillation vial containing a scintillation 'cocktail' prepared by mixing a solution of 12 ml of 4 M- NH_3 , 100 ml of Nuclear-Chicago solubilizer (Nuclear-Chicago, Chicago, IL, U.S.A.), and 1 litre of Liquifluor scintillation fluid (New England Nuclear). Radioactivity was measured in a Packard Tri-Carb scintillation counter (model 3310) with an efficiency of 35% for 3H and 90% for ^{14}C . The lowest 5 cm of the gels were not assayed, because no radioactivity was ever found in that region.

Polyacrylamide-gel data were processed on a Wang 2200 system programmable calculator. A program was devised that could be used to assemble the data for a dual-labelled gel, correct the 3H counts for

cross-over from the ^{14}C channel, compute the ratio of one radioactive isotope to the other for each gel slice, and statistically analyse the ratio values.

Results

Growth and growth inhibition of neuroblastoma cells

The mouse neuroblastoma N2a line had a generation time of 20–24 h (Fig. 1). When radioautography was carried out on growing cultures labelled for 30 min with [3H]thymidine, 50% of the nuclei appeared to be undergoing DNA synthesis (Fig. 2), an indication that S-phase is approx. 50% of the cell cycle, with a duration of 10–12 h.

To investigate the effects of growth changes on glycoprotein metabolism, we devised a method to inhibit the growth of these cells. After monitoring the effects of various concentrations of dibutyl cyclic AMP and theophylline on neuroblastoma cell growth, it was determined that a mixture of 1.0 mM-dibutyl cyclic AMP plus 1.5 mM-theophylline gave

Fig. 1. Growth and growth inhibition of neuroblastoma-N2a cells

Monolayer cultures at 95% confluency were treated with Viokase and replated in 60 mm-diameter plastic dishes at 10^6 cells per dish. After 2 days of growth (arrow), 1.0 mM-dibutyl cyclic AMP plus 1.5 mM-theophylline was added to half of the cultures and cell number was counted for several days of growth. Symbols: ●, incubated in complete medium; ○, incubated in complete medium plus 1.0 mM-dibutyl cyclic AMP plus 1.5 mM-theophylline. (The vertical bars indicate the range of duplicate cultures.)

Fig. 2. DNA synthesis in neuroblastoma cells after growth inhibition and reversal from growth inhibition as measured by radioautography

Plastic dishes (60mm diameter) containing glass coverslips were seeded with 0.5×10^6 cells. After 1 day in culture, the monolayer cultures were incubated in complete medium containing 1.0mM-dibutyryl cyclic AMP and 1.5mM-theophylline; 24h later, some of the cultures were re-fed with complete medium without dibutyryl cyclic AMP or theophylline. The arrow marks the time when the inhibitors were removed. Other cultures were re-fed with complete medium containing the inhibitors. The cultures were pulse-labelled with $2\mu\text{Ci}$ of $[^3\text{H}]$ thymidine for 30min, and radioautography was carried out as described in the Materials and Methods section. Symbols: ●, incubated in the presence of 1.0mM-dibutyryl cyclic AMP and 1.5mM-theophylline; ○, incubated in the absence of dibutyryl cyclic AMP and theophylline.

complete inhibition in 24–36h (Figs. 1 and 2). If cell number was determined, as in Fig. 1, it was found that, 36h after the cells had been incubated with the inhibitors, cell growth had ceased. If DNA synthesis was assayed by radioautography, it was found that, after 24h of incubation with the growth inhibitors, the percentage of cells in S phase had decreased from 55% to 12% and remained at that low value for 24–48h of further incubation (Fig. 2). Growth inhibition appeared to be reversible, for, if mono-

layer cultures that had been inhibited for 24h were washed and reincubated in complete medium without growth inhibitors, DNA synthesis immediately increased, reaching a peak 6h after reversal of inhibition at a value approx. 4-fold higher than that of inhibited cultures.

The rapidity with which the cells began DNA synthesis after release from inhibition would be consistent with blockage of a large population of the cells near the G1/S boundary of the cell cycle. If all the cells were stopped at that point and entered S phase immediately on removal of the inhibitor, however, one would expect DNA synthesis to reach a peak at 100% of the cells in S phase. Because the peak value was 48%, it would appear that about 50% of the cells belonged to this synchronized population. After reaching a peak at 6h, the percentage of cells in S phase declined at 10h and reached a minimum between 10 and 18h after reversal. This would be consistent with a value for the duration of the S phase of the cell cycle of approx. 12h as measured by radioautography.

Treatment of N2a-cell cultures with concentrations of up to 2mM-dibutyryl cyclic AMP or theophylline resulted in incomplete growth inhibition in 36h. Of the combinations tested of the two agents together, 1.0mM-dibutyryl cyclic AMP plus 1.5mM-theophylline was most readily reversible (results not shown). Thus, the latter was the chosen method for studying the effect of growth inhibition on plasma-membrane glycoprotein metabolism.

Membrane glycoprotein metabolism in intact cells

Membrane glycoprotein metabolism was assayed in intact cells by labelling with radioactive fucose, a sugar that is primarily incorporated into plasma membrane of eukaryotic cells (Bennett & Leblond, 1970; Bennett *et al.*, 1974; Atkinson, 1975). It has been shown previously that incorporation of fucose into membrane glycolipids is minimal in neuroblastoma N2a cells (Hudson & Johnson, 1977a) and that fucose is a good indicator of glycoprotein metabolism in these cells.

The incorporation of $[^3\text{H}]$ fucose into acid-insoluble glycoproteins was compared for growing and growth-inhibited cells (Fig. 3). In both cases it appeared that incorporation of fucose reached a steady state approx. 12h after addition of the radioactively labelled sugar. The two metabolic states differed, however, in that the growth-inhibited cells incorporated about half as much $[^3\text{H}]$ fucose into macromolecules as did growing cells.

To ascertain whether the apparent inhibition of glycoprotein synthesis was reversible, cultures that had been inhibited for 2h were re-fed with complete medium and labelled with $[^3\text{H}]$ fucose at the time that the inhibitors were removed. Growing cells labelled

Fig. 3. Kinetics of $[^3\text{H}]$ fucose incorporation into acid-insoluble fractions of intact neuroblastoma cells

Cells grown in 60mm-diameter dishes in complete medium or in complete medium containing dibutyryl cyclic AMP and theophylline were incubated with $1\mu\text{Ci}$ of $[^3\text{H}]$ fucose/ml, supplemented with $2.5\mu\text{M}$ -fucose. At the times indicated, duplicate dishes were assayed for acid-insoluble cellular radioactivity as described in the Materials and Methods section. Symbols: ●, cells grown in complete medium; ○, cells grown in complete medium containing 1.0mm-dibutyryl cyclic AMP and 1.5mm-theophylline. (Vertical bars indicate experimental ranges of duplicate cultures.)

with $[^3\text{H}]$ fucose served as controls (Table 1). The reversed cells, which had somewhat less than half the acid-insoluble specific radioactivity of the controls at 3.5h after reversal, recovered to more than 86% of the control specific radioactivity within 8h. These results indicated that the inhibition of both fucose incorporation and cell growth mediated by dibutyryl cyclic AMP and theophylline was readily reversible.

It was possible that the decrease in fucose incorporation in growth-inhibited cells was primarily the result of an inhibition of fucose uptake at the cell surface and not necessarily a reflection of true glycoprotein synthesis. To overcome this problem, uptake of $[^3\text{H}]$ fucose into acid-soluble pools was assayed in growing and growth-inhibited cells (Table 2). Similar to the incorporation of fucose into cell glycoproteins (Fig. 3), uptake reached a steady state at the same time in both growing and growth-inhibited neuroblastoma cells, but the inhibited cells took up half as

Table 1. Incorporation of $[^3\text{H}]$ fucose into glycoproteins by growing and growth-inhibited and reversed cells

Monolayer cultures grown in complete medium were incubated with $1\mu\text{Ci}$ of $[^3\text{H}]$ fucose/ml supplemented with $2.5\mu\text{M}$ -fucose and were assayed at subsequent time periods for acid-insoluble specific radioactivity as described in the Materials and Methods section. Control cells were incubated in complete medium without inhibitors. Reversed cells were treated with 1.0mm-dibutyryl cyclic AMP and 1.5mm-theophylline for 24h before $[^3\text{H}]$ fucose was added. The cells were then washed and incubated with $[^3\text{H}]$ fucose in medium lacking growth inhibitors. (Values represent means for duplicate samples $\pm$ range; those in parentheses are percentages of control specific radioactivity.)

Time after addition of $[^3\text{H}]$ fucose (h)	$10^{-4} \times$ Acid-insoluble radioactivity (c.p.m./mg of protein)	
	Control cells	Growth-inhibited and then reversed cells
3.5	1.144 ± 0.035	0.412 ± 0.001 (36.0)
5.5	1.505 ± 0.093	0.854 ± 0.029 (56.7)
8.0	1.912 ± 0.119	1.652 ± 0.083 (86.4)

Table 2. Uptake of $[^3\text{H}]$ fucose into acid-soluble pools of growing and growth-inhibited cells

Monolayer cultures grown in complete medium were incubated with $1\mu\text{Ci}$ of $[^3\text{H}]$ fucose/ml supplemented with $2.5\mu\text{M}$ -fucose and were assayed at subsequent time points for acid-soluble radioactivity as described in the Materials and Methods section. Control cells were incubated in complete medium with no additions throughout. Inhibited cells were treated with 1.0mm-dibutyryl cyclic AMP and 1.5mm-theophylline for 24h before $[^3\text{H}]$ fucose was added and throughout the labelling period. (Values represent the means of duplicate samples $\pm$ range.)

Time after addition of $[^3\text{H}]$ fucose (h)	$10^{-4} \times$ Acid-soluble radioactivity (c.p.m./mg of protein)	
	Control cells	Inhibited cells
3.5	0.823 ± 0.034	0.192 ± 0.007
5.5	1.01 ± 0.160	0.537 ± 0.039
8.0	0.966 ± 0.015	0.494 ± 0.011

much fucose. It therefore seemed that the apparent difference in incorporation between either of the two metabolic states, or the effects of dibutyryl cyclic AMP and theophylline, could be explained by an alteration of transport processes. That effect, however, was not immediate, because 1.0mm-dibutyryl cyclic AMP and 1.5mm-theophylline added to

growing cells with [^3H]fucose had no effect on fucose uptake and incorporation (results not shown).

Hudson & Johnson (1977b) have shown, with this same cell line, that fucosylated glycoproteins of the cell surface exhibit differential rates of degradation and/or turnover. When N2a-cell monolayer cultures were labelled to a steady state with [^3H]fucose (24 h), washed, incubated in complete medium without fucose and assayed for acid-insoluble radioactivity, an initial 20% decrease in specific radioactivity was observed within the first 3 h after the radioactive precursor had been removed (Fig. 4). Subsequent degradation then appeared to proceed more slowly. The initial rapid decrease in specific radioactivity of

Fig. 4. Degradation of fucosylated macromolecules in intact neuroblastoma cells

Growing and growth-inhibited cells were incubated with $1\mu\text{Ci}$ of [^3H]fucose/ml, supplemented with $2.5\mu\text{M}$ -fucose. The cells were then washed twice with non-radioactive medium and incubated in complete medium without fucose. At times indicated after the removal of fucose, duplicate dishes were assayed for acid-insoluble specific radioactivity by the procedure mentioned in the Materials and Methods section. Symbols: ●, cells grown in complete medium; ○, cells treated with 1.0mM -dibutyl cyclic AMP and 1.5mM -theophylline beginning 12h before addition of [^3H]fucose and during subsequent incubations. (Vertical bars indicate the experimental range of duplicate cultures.)

Fig. 5. Degradation of fucosylated macromolecules in intact neuroblastoma cells reversed from growth inhibition. Monolayer cultures were treated with 1.0mM -dibutyl cyclic AMP and 1.5mM -theophylline. After 12h, $1\mu\text{Ci}$ of [^3H]fucose/ml was added and supplemented with $2.5\mu\text{M}$ -fucose. The cells were washed after 24h of labelling with complete medium lacking both growth inhibitors and fucose, and the cells were reincubated in the absence of fucose and growth-inhibitors in complete media. At subsequent time points after reincubation, duplicate samples were assayed for acid-insoluble specific radioactivity as described in the Materials and Methods section. (Vertical bars indicate experimental values of duplicate cultures.)

cellular glycoproteins was attributed to a population of membrane glycoproteins that turned over rapidly (Hudson & Johnson, 1977b). In contrast with growing cells, cells that had been growth-inhibited for 36h and labelled with [^3H]fucose for 24h during inhibition showed no degradation within 6h. These results could have been evidence either that the rapidly degrading population of glycoproteins was not being synthesized in growth-inhibited cultures or that these glycoproteins, although synthesized, were not being preferentially degraded. To distinguish between these two possibilities, a reversal experiment was carried out. Cultures that had been growth-inhibited for 36h and labelled for 24h were reincubated in complete medium without radioactive fucose or growth inhibitors and assayed for degradation of

fucosylated macromolecules (Fig. 5). Similarly to growing cell populations, the specific radioactivity decreased by approx. 20% in the first 2h after [^3H]fucose and the inhibitors had been removed. These results indicated that the rapidly degraded population of membrane glycoproteins was present and was being glycosylated in growth-inhibited monolayer cultures, but was not being preferentially degraded.

Plasma-membrane experiments

To investigate specific membrane glycoprotein changes resulting from growth inhibition, a series of experiments was carried out by polyacrylamide-gel electrophoresis of neuroblastoma-N2a plasma membranes. As a preliminary experiment, plasma membranes isolated from monolayer cultures as described in the Materials and Methods section were separated on 5.6% sodium dodecyl sulphate/urea/polyacrylamide gels, which were then stained for protein with Coomassie Blue. Fig. 6 shows the A_{550} profile for a gel of membranes obtained from normally growing cells. Comparisons of membrane profiles of growing and growth-inhibited cells showed only slight differences; however, because profiles of two different membrane preparations from growing neuroblastoma cells yielded differences of the same magnitude, the differences observed between growing and inhibited

cells were not considered to be significant (results not shown). If differences existed between specific membrane glycoproteins of growing and non-growing cells, they were not discernible by staining.

Dual labelling with radioactive fucose, a more sensitive technique specific for membrane glycoproteins, was subsequently carried out. Monolayer cultures were incubated for 24h with the dibutyryl cyclic AMP/theophylline mixture and labelled with [^3H]fucose for an additional 10h in the presence of the inhibitors. Growing cultures were radioactively labelled for 10h with [^{14}C]fucose. Membranes were isolated from the growing and the inhibited cultures, mixed and electrophoresed on the same gel essentially as described by Mathews *et al.* (1976). The gel was sliced and the ratio of $^3\text{H}/^{14}\text{C}$ was determined for each slice (Hudson & Johnson, 1977b). In that no ratios appeared consistently outside the limits of variability from experiment to experiment (Fig. 7), it appeared that there was little or no difference in the relative synthesis of specific fucosylated membrane glycoproteins between growing and growth-inhibited cells. The one point appearing above the limits of variability in Fig. 7 did not appear outside the limits in other experiments. It is not unusual to find a single point outside the limits of variability, but these points were not consistent from one gel profile to another in that they were randomly distributed.

Fig. 6. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of plasma-membrane proteins

Neuroblastoma cells were grown on 100mm-diameter culture dishes in complete medium. Plasma membranes were isolated and 45 μg of protein was electrophoresed as described in the Materials and Methods section. The gels were stained for protein and scanned at 550nm as described. The molecular-weight scale was determined on the basis of gel electrophoresis of molecular-weight standards as described.

Fig. 7. Relative synthesis of glycoproteins from plasma membranes of growing and growth-inhibited cells

Monolayer cultures of 100mm-diameter dishes were incubated in complete medium containing 0.3 μCi of [^{14}C]fucose/ml for 10h. Other cultures were treated with 1.0mM-dibutyryl cyclic AMP and 1.5mM-theophylline for 24h, after which 3 μCi of [^3H]fucose/ml in 2.5 μM -fucose was added and the cells were incubated for an additional 10h. The labelled cells were combined and the membranes were isolated and electrophoresed as described in the Materials and Methods section. The gel was sliced and the $^3\text{H}/^{14}\text{C}$ ratio was determined for each slice. The broken lines represent the tolerance limits of variability where $P=0.05$ (Mathews *et al.*, 1976). The molecular-weight scale was determined on the basis of gel electrophoresis of molecular-weight standards as described.

Fig. 8. Turnover of fucosylated glycoproteins in neuroblastoma plasma membrane

Plasma membranes were isolated and electrophoresed, and radioactivity was determined as described in the Materials and Methods section. The broken lines represent the tolerance limits of variability where $P = 0.05$. (a) Cells were incubated for 24h in complete medium containing $0.25 \mu\text{Ci}$ of [^{14}C]fucose/ml, then washed twice with fresh media, reincubated for 6h in complete media without fucose, and then reincubated for 2.5h in complete medium containing $3 \mu\text{Ci}$ of [^3H]fucose/ml. (b) Cells were incubated for 12h in complete medium containing 1.0mM-dibutyl cyclic AMP and 1.5mM-theophylline, and all subsequent incubations were in the presence of these inhibitors. The cells were then labelled with $0.25 \mu\text{Ci}$ of [^{14}C]fucose/ml for 12h, washed twice, reincubated without fucose for 6h, and then reincubated in the presence of $3 \mu\text{Ci}$ of [^3H]fucose/ml. (c) Cells were incubated for 12h in complete medium containing 1.0mM-dibutyl cyclic AMP and 1.5mM-theophylline, after which $0.25 \mu\text{Ci}$ of [^{14}C]fucose/ml was added and incubated for 24h. The cells were washed twice and reincubated in complete medium containing the inhibitors for 4h, then the cells were reincubated in complete medium without growth inhibitors for 2.5h, and $3 \mu\text{Ci}$ of [^3H]fucose/ml was added and incubated for an additional 2.5h.

Turnover of membrane glycoproteins was assayed by labelling neuroblastoma cells for 24h with [^{14}C]fucose, incubating in medium containing no fucose for 6h to deplete intracellular radioactive fucose, and then radioactively labelling the cells with [^3H]fucose for 2.5h. The plasma membranes of cells labelled by this protocol were isolated, the glycoproteins were separated on sodium dodecyl sulphate/urea/polyacrylamide gels, and the $^3\text{H}/^{14}\text{C}$ ratios for each portion of the gel were computed. Fig. 8(a) shows the ratios depicting glycoprotein turnover in plasma membranes of growing cells. As noted previously (Hudson & Johnson, 1977b), heterogeneous rates of turnover are characteristic of growing N2a cells.

Turnover data for membrane glycoproteins from growth-inhibited cells were obtained by incubating monolayer cultures with the dibutyl cyclic AMP/theophylline mixture for 24h before labelling with radioactive fucose and for the entire dual-labelling period. The cells were labelled to a steady state with [^{14}C]fucose, incubated without fucose for 6h and reincubated with [^3H]fucose for 2.5h before membrane isolation and electrophoresis. Rapid turnover of high-molecular-weight glycoproteins, as found for growing cells, was not evident in the growth-inhibited cultures (Fig. 8b). If growth-inhibited cells were labelled with [^{14}C]fucose for 24h, the inhibitors removed and [^3H]fucose was added, the pattern of turnover in the cells more closely resembled that of growth-inhibited cells than of growing cells (Fig. 8c).

It appears therefore that, although protein degradation in intact cells growing and reversed from growth inhibition (Figs. 4 and 5) appeared similar, the reasons for the rapid kinetics of degradation were quite different. In growing cells preferential turnover rates for specific groups of plasma-membrane glycoproteins could be easily identified (Fig. 8). However, the alteration of glycoprotein metabolism after removal of dibutyl cyclic AMP and theophylline appeared to represent a more general change without preferential turnover of the specific membrane glycoproteins detected in growing N2a cells (Fig. 8).

Discussion

Treating neuroblastoma N2a cells with dibutyl cyclic AMP and theophylline to inhibit growth and reversing growth inhibition by removing the inhibitors did confer some degree of synchrony on these cells (Fig. 2). In that the number of cells synthesizing DNA was quite low before reversal and increased dramatically on release from inhibition, it would appear that a major population of the inhibited cells was trapped near the G1/S boundary and moved into S phase on removal of the inhibitors. Dibutyl cyclic AMP and phosphodiesterase inhibitors such as theophylline are known to arrest various cell types

(Greengard, 1978), including neuroblastoma cells (Prasad *et al.*, 1973), in the G1 phase. However, since DNA synthesis after release of growth inhibition did not reach 100% (Fig. 2), it would appear that not all of the cells were blocked at the G1/S boundary. An alternative explanation might be that not all of the cells experienced recovery immediately on removal of dibutyryl cyclic AMP and theophylline.

It is not likely that cell death is the reason that 100% of the cells failed to initiate DNA synthesis on reversal, since fucose incorporation seemed to recover (Table 1) and Trypan Blue exclusion showed no significant decrease in viability after 48 h of incubation with dibutyryl cyclic AMP and theophylline (results not shown).

Dibutyryl cyclic AMP/theophylline treatment of N2a-cell cultures apparently conferred two kinds of alterations on membrane glycoprotein metabolism: (1) changes in the overall degradation of glycoproteins (Fig. 4); and (2) changes in turnover of a specific class of glycoproteins of apparent mol.wt. greater than 100000 (Fig. 8). A decrease in apparent fucose incorporation into glycoproteins could also be observed in intact cells on growth arrest; however, that finding could be accounted for by decreased uptake of [^3H]fucose and might not represent decreased synthesis of fucosylated glycoproteins.

The loss of rapid degradation of glycoproteins after growth arrest, as measured in intact cells, was readily reversible on reincubation of arrested cells in medium without inhibitors (Figs. 4 and 5). That would indicate that this population of glycoproteins was being synthesized in inhibited cells, but degraded rapidly only in growing cultures. Similarly, Rieber *et al.* (1975), with a virus-transformed rat kidney-cell line, found that the 250000-mol.wt. (LETS) membrane glycoprotein was more stable in confluent non-transformed cultures than in growing or transformed cells, where it is degraded to smaller glycopeptides. That was also consistent with our finding that turnover of high-molecular-weight glycoproteins is more rapid in growing than in growth-inhibited cells (Fig. 8).

The results in Fig. 8 show no significant recovery in turnover of high-molecular-weight membrane glycoproteins in cells reversed from growth inhibition 2.5–5 h after removal of the growth inhibitors. The absence of rapid turnover in these glycoprotein peaks after reversal could be explained by the fact that, although degradation of fucosylated glycoproteins had recovered by this time (Fig. 5), incorporation of [^3H]fucose into these glycoproteins had not (Table 1). Degradation without resynthesis would also tend to decrease the ^{14}C radioactivity without changing that of ^3H , resulting in increased $^3\text{H}/^{14}\text{C}$ ratios. Indeed, the mean ratio for reversed cells did appear to be higher than for growing cultures (Fig. 8).

Examination of the turnover patterns in Fig. 8

more closely reveals slight differences in the turnover of the low-molecular-weight glycoproteins among growing, growth-inhibited and reversed cell-membrane glycoproteins. It is difficult to assign significance to these differences, however, because the actual glycoprotein patterns had relatively low radioactivity in the low-molecular-weight region of the gels. The difference observed in turnover of the high-molecular-weight glycoproteins, however, occurred not only in an area of the gel of high radioactivity, but also was consistent from one gel to another.

In the neuroblastoma system, there was a decrease of approx. 50% in the incorporation of exogenous [^3H]fucose into acid-insoluble glycoproteins in growth-inhibited cells (Fig. 3) and an increase in apparent incorporation in cells that had been reversed from growth arrest for 8 h (Table 1). However, the decrease in measured incorporation could be accounted for by growth-dependent changes in uptake of fucose into acid-soluble pools (Table 2). Because a major population of growth-inhibited cells appear to be in very late G1 phase and appeared to be in S phase 8 h after reversal, these changes in uptake of fucose could reflect differences in membrane transport of fucose at different stages of the cell cycle. Uptake and incorporation of fucose into membrane glycoproteins has been shown to be increased in late S phase in HeLa cells (Nowakowski *et al.*, 1972). Several other investigators, with mammalian cell lines, have also observed increases in incorporation of sugars, including fucose, into membrane glycoproteins at one particular phase of the cell cycle (Bosmann & Winston, 1970; Gerner *et al.*, 1970; Graham, 1973). Unfortunately, the soluble pools were not monitored in these cases, and it was not possible to determine if the results reflected changes in uptake of the radioactive sugars or an actual alteration in macromolecular metabolism.

It is true that cyclic AMP, through its activation of cyclic AMP-dependent protein kinases, mediates other effects as well as inhibiting cell proliferation and trapping cells in the G1 phase of the cell cycle (Greengard, 1978). It is possible that dibutyryl cyclic AMP and theophylline directly inhibited cellular proteinases and decreased glycoprotein degradation; cyclic AMP also could have changed membrane glycoprotein metabolism by inhibiting protein synthesis at the level of translation. However, in that growth-dependent alterations in membrane glycoprotein metabolism and, specifically, turnover have been previously documented (Bosmann & Winston, 1970; Gerner *et al.*, 1970; Nowakowski *et al.*, 1972; Graham, 1973; Kaplan & Moskowitz, 1975; Rieber *et al.*, 1975; Truding *et al.*, 1975), it would be reasonable to assume that the growth-inhibitory effects of dibutyryl cyclic AMP and theophylline are the major cause of these alterations in N2a cells. The close correlation between reversal of growth inhibition,

the recovery of glycoprotein degradation, and the recovery of apparent fucose incorporation also lend credence to the relationship of cyclic AMP-dependent alterations in neuroblastoma plasma-membrane metabolism to cell growth.

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References

- Atkinson, P. H. (1975) *J. Biol. Chem.* **230**, 2123-2134
- Barbarese, E., Sauerwein, H. & Simpkins, H. (1973) *J. Membr. Biol.* **13**, 129-142
- Bennett, G. & Leblond, C. P. (1970) *J. Cell Biol.* **46**, 409-416
- Bennett, G., Leblond, C. P. & Haddad, A. (1974) *J. Cell Biol.* **60**, 258-284
- Bosmann, H. B. & Winston, R. A. (1970) *J. Cell Biol.* **45**, 23-33
- Burger, M. M. (1970) *Nature (London)* **227**, 170-171
- Burger, M. M. (1971) *Biomembranes* **2**, 247-270
- Chou, I.-N., Black, P. H. & Roblin, R. O. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1748-1752
- Collard, J. G. & Smets, L. A. (1974) *Exp. Cell Res.* **86**, 75-80
- Cunningham, D. D., Thrash, C. R. & Ho, T.-S. (1975) in *Molecular Aspects of Membrane Phenomena* (Kabeck, H. R., Neurath, H., Radda, G. K., Schwyzer, R. & Wiley, W. R., eds.), pp. 132-148, Springer-Verlag, Berlin
- Dehlinger, P. J. & Schimke, R. T. (1971) *J. Biol. Chem.* **246**, 2574-2583
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1748-1752
- Frank, W. (1972) *Exp. Cell Res.* **71**, 238-241
- Froehlich, J. E. & Rachmeler, M. (1972) *J. Cell Biol.* **55**, 19-31
- Gahmberg, C. G. & Hakomori, S. (1974) *Biochem. Biophys. Res. Commun.* **59**, 283-291
- Gerner, E. W., Glick, M. C. & Warren, L. (1970) *J. Cell. Physiol.* **75**, 275-280
- Glick, M. C., Rabinowitz, Z. & Sachs, L. (1974) *J. Virol.* **13**, 967-974
- Graham, J. M. (1973) *Nature (London)* **246**, 291-295
- Greengard, P. (1978) *Science* **199**, 146-152
- Hudson, J. E. & Johnson, T. C. (1977a) *Biochim. Biophys. Acta* **497**, 567-577
- Hudson, J. E. & Johnson, T. C. (1977b) *Biochem. J.* **166**, 217-223
- Hynes, R. O. & Bye, J. M. (1974) *Cell* **3**, 113-120
- Kaplan, J. & Moskowitz, M. (1975) *Biochim. Biophys. Acta* **389**, 306-313
- Landry, J. & Marceau, N. (1975) *Biochim. Biophys. Acta* **389**, 154-161
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Mathews, R. A., Johnson, T. C. & Hudson, J. E. (1976) *Biochem. J.* **154**, 57-64
- Nowakowski, M., Atkinson, P. H. & Summers, D. F. (1972) *Biochim. Biophys. Acta* **266**, 154-160
- Ozanne, B. & Sambrook, J. (1971) *Nature (London)* **232**, 156-160
- Prasad, K. N. & Sheppard, J. R. (1972) *Proc. Soc. Exp. Biol. Med.* **141**, 240-246
- Prasad, K. N., Kumar, S., Gilman, K. & Vernadakis, A. (1973) *Biochem. Biophys. Res. Commun.* **50**, 973-977
- Rieber, M., Bacalao, J. & Alonso, G. (1975) *Cancer Res.* **35**, 2104-2108
- Sheppard, J. R. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1316-1320
- Shin, B. C., Ebner, K. E., Hudson, B. G. & Carraway, K. C. (1975) *Cancer Res.* **35**, 1135-1140
- Shoham, J. & Sachs, L. (1974) *Exp. Cell Res.* **85**, 8-14
- Truding, R., Shelanski, M. L. & Morrell, P. (1975) *J. Biol. Chem.* **250**, 9348-9354
- Tweto, J. & Doyle, D. (1975) *J. Biol. Chem.* **251**, 872-882
- Warren, L., Glick, M. C. & Nass, M. K. (1966) *J. Cell. Physiol.* **68**, 269-287
- Warren, L., Fuhrer, J. P. & Buck, C. A. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 80-85