C-1300 murine neuroblastoma cells release glycoproteins into the culture medium. The process was studied by prelabeling spinner cultures for 12 to 60 hours with \(^{13}C\) glucosamine. Then, the medium was removed and replaced with fresh medium lacking radioactive isotope. Soluble material released into the medium during the subsequent 2-hour incubation was collected by trichloroacetic acid precipitation. The released proteins were then separated by discontinuous polyacrylamide gel electrophoresis in buffers containing sodium dodecyl sulfate. The electrophoretograms of glycoproteins obtained from cultures labeled for different lengths of time were very similar; three major radioactive regions centered about molecular weights 87,000, 66,000, and 55,000 were present.

When spinner cells were transferred to monolayer culture in the presence of \(N^\ominus, O^\oplus\) dibutyryl adenosine 3':5'-monophosphate (Bt,cAMP), differentiation (extension of neurites twice the diameter of the perikaryon) was observed. Monolayer cultures grown in the presence of Bt,cAMP and \(^{3}H\) glucosamine for 12 hours released glycoproteins which gave a gel electrophoresis pattern similar to that obtained using spinner cultures. However, after 60 hours in the presence of Bt,cAMP and \(^{3}H\) glucosamine, the released radioactive material consisted almost exclusively of glycoproteins of the 66,000 molecular weight class. Similar results were obtained if \(^{14}C\) glucose was substituted for \(^{3}H\) glucosamine, or if bromodeoxyuridine (which also induced differentiation) was substituted for Bt,cAMP.

Similar experiments using radioactive amino acids were conducted with both spinner and monolayer cultures. Much of the released radioactive material was contained in the same three molecular weight classes as the glycoproteins released by spinner cells prelabeled with \(^{3}H\) glucosamine, and this pattern did not vary with length of labeling period or type of culture. These results may imply that the glycosylation of released proteins is influenced by agents which can induce differentiation. The origin of this released material is discussed.

\(^{14}C\) Glucosamine-labeled glycoproteins of the molecular weight class centered about 55,000 (discussed above) were isolated by preparative gel electrophoresis. They co-migrated with authentic mouse brain microtubular protein as two closely spaced bands on a number of different electrophoretic systems. This protein fraction was also characterized as complexing with a monospecific antitubulin antibody.

Glycoproteins of the surface membrane are presumed to play an important role in controlling the interactions between cells in culture (1-3). Some investigations of the glycoprotein material released into the culture medium of metabolically active cells have been reported (4-15). This released or "shed" material could be a product of the normal metabolic turnover of surface membrane glycoproteins, or possibly be a specifically released cytoplasmic material involved in the regulation of cell to cell interactions. As an extension of our interest in membranes of the nervous system, we utilized such an approach to study the differentiation of cultured neuroblastoma cells.

The C-1300 murine neuroblastoma propagated at the Jackson Laboratories can be cultured, cloned, and maintained in a "undifferentiated" form (16-17). Under certain physiological conditions, these cells can be induced to extend neurites concomitant with the induction of some enzyme activities characteristic of mature neurons (18-20). We have
demonstrated certain changes in the glycoprotein composition of the surface membrane and soluble cytoplasmic proteins in the course of this differentiation (21). In the course of these studies, we observed that if radioactive glucosamine was used to label cells, some of the radioactivity then became associated with the soluble extracellular macromolecules. We have found that the release of glycoproteins into the culture medium by neuroblastoma cells is a specific process and is related to the state of differentiation of the cell.

**EXPERIMENTAL PROCEDURE**

**Cell Culture**—A neuroblastoma line N2aE (obtained from Drs. F. Ruddle and R. Rosenbaum of Yale University as N2a) was adapted to spinner culture (21). Stock cultures were maintained in suspension with a spin bar in an Erlenmeyer flask at 37°C. The density was adjusted daily to 5 x 10^4 cells/ml by dilution with fresh spinner culture medium (Eagle’s Minimum Essential Medium for suspension culture (GIBCO) supplemented with 5% calf serum, 5% fetal calf serum, 2 mM glutamine, 95 units/ml of penicillin G, and 10 mg/ml of streptomycin sulfate). For growth of monolayer cells, a monolayer culture medium (Eagle’s Minimum Essential Medium for monolayer cultures, supplemented as above) was used, and the cells were fed every 2 days.

Experiments with spinner cultures were initiated by the addition of [6-3H]glucosamine (0.5 μCi/ml at 5 to 10 Ci/mM), [1-3H]glucosamine (0.05 μCi/ml at 45 to 55 Ci/mM), or [6-3H]glucose (7 μCi/ml at 10 to 15 Ci/mM), L-[3,4,5-3H]glucosamine (1 μCi/ml at 5 Ci/mM), or L-[2-3H]glucose (0.2 μCi/ml at 42 Ci/mM), all from New England Nuclear. In some cases, the spinner cultures were supplemented with either 1 x 10^-3 M Bt,cAMP (Research Plus or Sigma), 1 x 10^-3 M bromodeoxyuridine (Calbiochem), or 5 x 10^-4 M sodium butyrate (Sigma). After the stated time (12 to 60 hours) (including a dilution with fresh radioactive medium every 24 hours for the longer time point), cells were washed in serum-free spinner culture medium by three cycles of centrifugation at 130 x g for 4 min. The resultant supernatant was then resuspended to one-fifth of the original volume with fresh serum-free spinner culture medium lacking a radioactive isotope. If the cells were originally grown in Bt,cAMP or bromodeoxyuridine, the subsequent 2-hour incubation also contained this reagent. Following 2 hours in this suspension culture, the cells were removed from the medium by centrifugation at 130 x g for 10 min. Viability of the cells through this step was greater than 95% as assayed by trypan blue exclusion. The supernatant was then centrifuged at 130,000 x g for 60 min. The extracellular soluble proteins in the high speed supernatant were collected by overnight precipitation in 10% trichloroacetic acid at 4°C. The precipitate was washed with 10% trichloroacetic acid at 4°C, and is related to the state of differentiation of the cell.

**Incorporated Sugar**

| Sugar | Incorporation | Materials |
|-------|--------------|-----------|
| Glucosamine | 9349 cpm | 100 pg of protein in 300 μl of protein solvent were applied to each lane. |
| Leucine | 36,000 cpm | Polyacrylamide Gel Electrophoresis—The released protein was routinely analyzed in a discontinuous polyacrylamide gel electrophoresis system (33 spacer gel/10% running gel) in buffers containing 0.1% sodium dodecyl sulfate (System a) (21, 22). About 100 μg of protein in 300 μl of protein solvent were applied to each gel. In the experiment characterizing the presumptive tubulin, a discontinuous gel (2.5% spacer gel/7.5% running gel) in buffers containing 8 M urea, but no detergent, was utilized as a preparative gel (System b) (23, 24). The bands of interest (Region c) were cut out, and the protein eluted and characterized by co-electrophoresis with authentic tubulin on System a, and on analytical gels similar to those of System b, but also containing 0.1% sodium dodecyl sulfate (System c) (24). This material was also analyzed on isoelectric focusing gels on a pH 3 to 10 gradient in 7% polyacrylamide (System d) (24, 25). Molecular weights were obtained by calibration with a protein standards kit (Schwarz/Mann).

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**RESULTS**

**Cell Growth**—Details regarding the growth of this cell line under our conditions have been published (21). In the present studies, cells in the logarithmic phase of growth had a doubling time of 18 hours. Monolayer cells in the presence of Bt,cAMP grew to a confluent monolayer with a doubling time of 30 hours during the logarithmic growth phase. Within a few hours of transfer, most of the cells had flattened, and many of the cells had started to extend neurites. By 24 hours, a majority of the cells had extended neurites; and by 60 hours, about 75% of the cells were differentiated (i.e. had extended neurites at least twice as long as the perikaryon diameter). Monolayer cultures treated with bromodeoxyuridine were similarly differentiated. Treatment with sodium butyrate caused the cells to adhere to the substratum and slowed the growth rate but did not cause morphological differentiation. Spinner cultures, regardless of drug treatment, and untreated monolayer cultures retained an undifferentiated morphology.

**Release of Incorporated Sugar Label**—Monolayer and spinner cells were incubated for 12, 36, or 60 hours in the presence of radioactive glucosamine. The material released during the subsequent 2-hour incubation, in fresh serum and label-free medium, was isolated and subjected to polyacrylamide gel electrophoresis. In a typical experiment where the monolayer cells were prelabeled with 10 μCi of [3H]glucosamine for 36 hours, the distribution of trichloroacetic acid-insoluble radioactivity was: surface membrane, 45,000 cpm; soluble cytoplasmic proteins, 36,000 cpm; and released materials, 22,000 cpm. Correcting for yield (assuming 65% for surface membrane (21), 95% for cytoplasmic proteins, the extent of cell breakage (21)), and 100% for released protein, the distribution of

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1The abbreviation used is Bt,cAMP, N\[^1-\]O\[^\]^\,-dibutyryl adenosine 3',5'-monophosphate.
trichloroacetic acid-insoluble radioactivity was: surface membranes, 38% soluble cytoplasmic protein, 20%; released material, 12% with the remaining radioactivity being in the nuclear and debris fractions. Fig. 1 shows the electrophoretic analyses of glucosamine-labeled proteins released into the culture medium of Bt,cAMP-treated cells during differentiation. At the 12- and 36-hour time points, the released proteins were grouped into three major molecular weight regions centered about apparent molecular weight of about a, 87,000; b, 66,000; and c, 55,000; respectively (Fig. 1A). Each of these classes of proteins was defined by its position on the gel: Region a (gel slices 10 to 15 ± one slice); Region b (gel slices 18 to 23 ± one slice); or Region c (gel slices 24 to 31 ± one slice). Each class was probably composed of more than one glycoprotein; Region c was usually resolved into two bands. There was some labeled material at the origin (gel slice 1), as well as a small peak of radioactivity (gel slices 54 to 56) just before the dye front (gel slice 57 ± two slices). The gel pattern was not affected by extraction of the protein with chloroform/methanol, 2:1, v/v prior to electrophoresis. The relative distribution of radioactivity between the major glycoprotein classes changed with increasing time of culture in the presence of Bt,cAMP. In a series of eight time course experiments, the time of onset of this preferential appearance of Region b glycoprotein was variable. However, in each case, by 60 hours, primarily Region b glycoprotein was being released into the medium (Fig. 1C). There were no further marked changes in the pattern in the subsequent 24-hour period (Fig. 2A). If fucose was used to label the glycoproteins, the same development pattern was observed, resulting in glycosylation of primarily one molecular weight class (Region b) of released glycoproteins after 60 hours in Bt,cAMP (Fig. 2B). Another agent which induced morphological differentiation, bromodeoxyuridine, also induced a time dependent increase in the proportion of released label present as Region b glycoprotein (Fig. 2B). If a monolayer culture was treated with Bt,cAMP for 48 hours, and then radioactive glucosamine was added for 12 hours, the gel pattern of the protein released in a subsequent 2-hour incubation (in serum-free medium) was the same as that of Fig. 1C.

The change in pattern of released glycoprotein in Bt,cAMP-treated cells was not primarily a function of cell density. Monolayer cultures were plated as several different concentrations of cells, so that following a 60-hour prelabeling period with [3H]glucosamine in the presence of Bt,cAMP, different subconfluent densities were reached on the flasks. The gel patterns in each case were similar to those of Fig. 1C.

Monolayer cultures not treated with any drug, or treated with sodium butyrate, did not differentiate, and the electrophoretogram of released glycoproteins did not show an increase in the proportion of radioactivity in Region b. The lack of response to sodium butyrate (which slows growth) demonstrated that the specific change in the pattern of released glycoproteins induced by Bt,cAMP or bromodeoxyuridine was not due only to the slower growth rate observed in the presence of these drugs. Neither the presence of serum during the 2-hour release period nor extensive extra washes with serum-free Minimum Essential Medium, serum-containing medium, or 0.001 M EDTA in Ca*+- and Mg*+-free medium affected the electrophoretic pattern or subcellular distribution of radioactivity.

The results with the differentiating monolayer cultures were in contrast to the result obtained in spinner culture in the absence of Bt,cAMP (Fig. 3). In spinner cultures labeled for 12, 36, or 60 hours, the same three major molecular weight groupings in Regions a, b, and c were recognizable in the polyacrylamide gel pattern. The ratio of label incorporated in the three major glycoprotein regions did not change.
molecule was obtained by demonstrating that following differ-
entiation. The difference is much more dramatic after 60 hours of dif-

erentiating monolayer cells (Fig. 1C) much more than the results obtained using untreated spinner culture cells (Fig. 3C).

The differences between spinner and monolayer cultures in
molecular weight distribution of radioactivity incorporated into
released proteins was observable only when a sugar label was
utilized. When [3H]leucine was used to prelabel spinner culture cells and Bt,cAMP-treated monolayer cells from 12 to 60 hours, no marked differences were observed in the elec-
rophoretograms of glycoproteins released into the culture me-
dium in the subsequent 2-hour period. Fig. 4 illustrates the
results from the 60-hour time points (the difference in position
of the leading peak at slices 53 and 55 was not reproducible
and was due to gel variation). Similar results were obtained
when a mixture of 3H-amino-acid was used instead of leucine
alone. The electrophoretic pattern of the leucine-labeled re-
leased proteins consisted largely of the same three molecular
weight classes of proteins obtained from glucosamine labeled
cells (Fig. 5). One observable difference was the presence of
another peak, gel slice 36 of Fig. 5, in the leucine-labeled
proteins.

The experiments represented in Figs. 1 to 4 were repeated
with 2.5% calf serum and 2.5% fetal calf serum present during
the 2-hour release period. The resulting electrophoretic pat-
terns were very similar to those obtained when no serum was
present during the release period.

The release of material into the culture medium during the
2-hour release period was not due to nonspecific leakage of
cell-soluble proteins through the surface membrane. Fig. 6
illustrates the more complex pattern of soluble cytoplasmic glycoproteins relative to released glycoproteins. The
difference is much more dramatic after 60 hours of dif-
ferentiation.

Evidence that the radioactive sugar was on a glycoprotein
molecule was obtained by demonstrating that following di-

gestion with the proteolytic enzyme Pronase (0.7 mg/ml for
24 hours at 37°), material originally migrating in Regions a,
b, and c formed a smear in the low molecular weight regions
of the polyacrylamide gels. Following limited acid hydrolysis
of the [3H]glucosamine-labeled released glycoprotein, more
than 95% of the radioactivity was eluted with glucosamine or
galactosamine from an amino acid analyzer. Also, substituting
4C]glucosamine for [3H]glucosamine did not change the gel
electrophoretic patterns. The label was not on a mucopolysac-
charide, since, when cells were grown in the presence of
SO4 and a delipidated membrane protein fraction prepared
and subjected to electrophoresis, no radioactivity migrated with
Regions a, b, or c.

Relationships between Released Glycoproteins and Surface Membranes Glycoproteins—The appearance of glycoproteins
in the culture medium may be related to metabolic turnover
of the surface membrane. Fig. 7 demonstrates the similarity
of the high molecular weight glycoproteins in both fractions
(Region a released glycoprotein and the surface membrane
glycoprotein peak centered about gel slice 13). However,
there is relatively little material in the surface membrane
glycoproteins corresponding to Regions b and c of the re-
leased glycoproteins.

Characterization of Region c Glycoprotein—The glycopro-
tein of Region c (usually resolved as two peaks) had an ap-
parent molecular weight centered at 55,000. If spinner

definition
glycoprotein precursors might arise from (a) labeling of serum neuroblastoma cells grown in the presence of radioactive against cell surface material released by HeLa cells inhibited 9352. It is also relevant to note that antibodies prepared normal cells with respect to proteolytic activity at the cell division of these cells (36).

These glycopeptides may be related to the glycoproteins released during normal growth of these cells (30-33). These glycopeptides may be released during normal growth of cells (12). Viral transformation of normal cells results in changes in the chromatographic properties of the surface peptides released by treatment with proteolytic enzymes (34). It has been suggested that transformed cells differ from normal cells with respect to proteolytic activity at the cell surface (35). It is also relevant to note that antibodies prepared against cell surface material released by HeLa cells inhibited division of these cells (36).

The radioactive glycoproteins found in the culture medium of neuroblastoma cells grown in the presence of radioactive glycoprotein precursors might arise from (a) labeling of serum proteins by cell surface enzymes; (b) leakage of intracellular material due to cell damage or death; (c) products of surface membrane metabolism; or (d) specific secretion of certain intracellular proteins. The first possibility is unlikely, since all of the peaks were labeled with leucine, and the addition of amino acids at the cell surface has not previously been reported. Also, if serum or bovine serum albumin was added during the 2-hour release period, neither the amount, nor gel electrophoretic pattern of the released glucosamine-labeled material was changed. The second possibility is unlikely because of the simplicity of the gel electrophoretic pattern of released glycoproteins (Fig. 6). Also, note the marked effect of Bt-cAMP which should not influence a nonspecific mechanism (Fig. 2C).

The likelihood that released proteins represent material "shed" from the surface membrane because of normal metabolic turnover has been suggested by several investigators (12, 37, 38). If this is the case, it is clear that not all surface membrane proteins are being shed intact at the same rate. Only the proteins of Region c were and Region c-released material (—) from spinner cultures (isolated on a preparative gel) and ^14C-labeled tubulin (— —). The isoelectric focusing gels contained sodium dodecyl sulfate and urea (System c). One of three experiments with similar results.

To test further for the possibility that this double peak in Region c was composed of tubulin, carrier tubulin (28) was added to the preparation and then precipitated with monospecific antitubulin (29). This gave a quantitative precipitation of the carrier and precipitated between 8 and 10% of the total counts in [H]glucosamine-labeled preparations. Electrophoresis of the precipitate, after dissociation in sodium dodecyl sulfate and urea (System c), showed over 90% of the radioactivity co-migrating with tubulin. Controls done with anticytofilament antiserum (cytofilament = 9 nm of filament) resulted in the precipitation of less than 0.5% of the counts, and these were widely dispersed on gel electrophoresis.

**FIG. 7.** Electrophoretogram of [^1^C]glucosamine-labeled released material (—) and [H]glucosamine-labeled surface membranes (—) from Bt-cAMP treated monolayer cultures labeled for 36 hours. The released material and surface membranes were isolated separately, solubilized, aliquots of each preparation were combined, and then subjected to electrophoresis. One of two similar experiments with similar results.

**FIG. 8.** Electrophoretogram of 36-hour [H]glucosamine-labeled Region C-released material (——) from spinner cultures (isolated on a preparative gel) and ^14C-labeled tubulin (— —). The discontinuous polyacrylamide gel contained both sodium dodecyl sulfate and urea (System c). One of three experiments with similar results.

**FIG. 9.** Electrophoretogram of 36-hour [H]glucosamine-labeled Region C-released material (——) from spinner cultures (isolated on a preparative gel) and ^14C-labeled tubulin (— —). The discontinuous polyacrylamide gel contained both sodium dodecyl sulfate and urea (System c). One of three experiments with similar results.

**FIG. 10.** Electrophoretogram of 36-hour [H]glucosamine-labeled Region C-released material (——) from spinner cultures (isolated on a preparative gel) and ^14C-labeled tubulin (— —). The discontinuous polyacrylamide gel contained both sodium dodecyl sulfate and urea (System c). One of three experiments with similar results.
weights of approximately 95,000, 70,000, and a double peak in serum-free medium. The gel pattern obtained in that study differentiated. Schubert (42) has studied the release of proteins shows major peaks corresponding to apparent molecular (analyzed by polyacrylamide gel electrophoresis) was studied under different conditions.

It is likely that the fourth possibility listed, specific secretion of certain soluble cytoplasmic components, also accounts for some of the released material. Region c-released glycoprotein corresponds in molecular weight to a double peak observed in the soluble cytoplasmic proteins (Fig. 6), and is not present as a major component of the surface membranes.

It has previously been shown that treatment of Chinese hamster ovary cells with Bt,cAMP can cause changes in glycosylation of the released proteins (15). We have shown that Bt,cAMP or bromodeoxyuridine induces changes in the pattern of molecular weight distribution of revealed glycoproteins of neuroblastoma cells. This may be due to relative differences in glycosylation of different proteins under different culture conditions. Another possible interpretation of the data is that there is a change in the relative rates of release of the three classes of glycoproteins under different conditions. This hypothesis is less likely because the distribution of leucine-labeled released proteins does not change with time; although if glycosylated proteins comprised only a small percentage of the total released proteins, changes specific to the glycoproteins might not be observed with leucine as a label. In monolayer culture, application of Bt,cAMP or bromodeoxyuridine caused a marked morphological differentiation along with induction of characteristic neuronal enzymes (18-20). Under certain conditions where the cells do not differentiate morphologically, these agents can still cause an increase in certain enzyme activities, i.e. biochemical differentiation (21, 39). The increase in the proportion of radioactivity released as Region b glycoprotein by spinner culture cells (Fig. 2C) upon application of Bt,cAMP is, therefore, not incompatible with this glycoprotein being associated with morphological differentiation.

Other relevant investigations of surface proteins in neuroblastoma cells include our previous work (21), showing that preferential appearance of a glycoprotein of molecular weight 105,000 in the soluble cytoplasm and surface membrane of neuroblastoma cells is induced under the same conditions which resulted in an increase in the proportion of radioactivity released as Region b glycoproteins into the release medium. Brown (40) demonstrated the appearance of a trypsin-released surface glycopeptide present in bromodeoxyuridine-treated cells but not in untreated cells. Glick and co-workers (41) have demonstrated differences in the chromatographic pattern of trypsin-released peptides from surfaces of various neuroblastoma clones, differing in their ability to differentiate. Schubert (42) has studied the release of proteins from neuroblastoma cells labeled with leucine for 24 hours in serum-free medium. The gel pattern obtained in that study shows major peaks corresponding to apparent molecular weights of approximately 95,000, 70,000, and a double peak centered at molecular weight 50,000, similar to those reported in this investigation. It was also noted that upon addition of [3H]leucine into the culture medium, the cells incorporated radioactivity into trichloroacetic acid-insoluble material almost immediately, but there was about an hour lag period before there was significant incorporation into extracellular protein.

Whatever the reason for shedding or secretion of glycoproteins in neuroblastoma cells, the data presented here demonstrate that this is a relatively specific phenomenon not due to random damage of cells. Furthermore, release of such glycoproteins, and possibly, their glycosylation, is under a highly specific physiological control.

Our results indicate that Region c glycoproteins released by the spinner culture co-migrate with tubulin upon electrophoresis. The observed molecular weight and isoelectric point relationship between tubulin and Region c-released glycoproteins might be coincidental, but definitive proof awaits further investigations. A preliminary experiment (in collaboration with Dr. H. Wainewski of the Albert Einstein College of Medicine) where vinblastine was added to the release medium revealed an ordered ultrastructure, similar to that seen when tubulin is precipitated by vinblastine (43). Tubulin has been found in neuroblastoma cells (44) and is involved in neurite formation (45). It has been reported that the concentration (per mg of protein) of intracellular tubulin is the same in differentiated and undifferentiated neuroblastoma cells (46, 47). Our observation of a specific release of a presumptive glycosylated tubulin into the culture medium might then imply a difference in metabolism of tubulin in undifferentiated, as compared to differentiated, neuroblastoma cells. The incorporation of glucosamine into this material is noteworthy. Although it has been reported by several laboratories that tubulin is a protein containing about 1.3% carbohydrate (48-50), this assumption has been challenged (51). The study of the metabolism of this material in subcellular and extracellular fractions of neuroblastoma cells under various physiological conditions may provide information relevant to the specialized role of microtubules in neurons.

It is also pertinent to note that actin-like proteins are found in brain cells (52, 53), cultured nerve cells (54), on the external surface of fibroblasts (55), and localized near the surface membrane of neuroblastoma cells (56, 57). Some of these actin-like proteins co-migrate with muscle actin on sodium dodecyl sulfate-containing gels, and have been reported to have a molecular weight of about 45,000 (54, 58), similar to the [3H]leucine-labeled protein centered about gel slice 33 of Fig. 4.

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