Regulation of Glycogen Metabolism in Astrocytoma and Neuroblastoma Cells in Culture

JANET V. PASSONNEAU AND SANDRA K. CRITES

From the Laboratory of Neuropathology and Neuroanatomical Sciences, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20014

The regulation of glycogen metabolism in C-6 astrocytoma and C-1300 neuroblastoma cells in culture has been investigated. Two modes of control of glycogen metabolism appear to be operative. The regulation of intracellular glycogen concentrations and the predominant forms of glycogen phosphorylase and glycogen synthase vary with (a) the available energy supply, and (b) altered intracellular concentration of cyclic adenosine 3':5'-monophosphate (cyclic AMP). Both cell lines respond to glucose in the medium; when glucose levels are high, glycogen is synthesized, glycogen phosphorylase a decreases, and glycogen synthase a increases. When glucose in the medium decreases to a critical level, the phosphorylase a increases and glycogen concentrations in the cells decrease in parallel with the medium glucose. The critical glucose concentration is 2.5 mM for the astrocytoma cells and 4 mM for the neuroblastoma cells. Insulin promotes the conversion of phosphorylase to the b form and synthase to the a form in both cell lines. All of these changes occur without alteration in the intracellular cyclic AMP concentrations. When cyclic AMP concentrations are increased in either cell line, phosphorylase a is increased, synthase a is decreased, and glycogen concentrations decrease. Isobutyl methylxanthine is effective in promoting glycogenolysis in both cell lines. Norepinephrine is effective with the astrocytoma cells, and prostaglandin E1 is effective with the neuroblastoma cells.

Several investigations have been made of glycogen metabolism in brain, with regard to the effect of various experimental procedures on glycogen turnover, accumulation, and degradation (1-6). One of the considerable problems in evaluating results observed with whole brain is that both neuronal and glial elements are involved. This is particularly important when one is considering glycogen metabolism, since many investigators consider that the glycogen is largely confined to glial elements, and particularly astrocytes (7). However, it has been shown in analyses of single cells that glycogen is present in at least some of the large neurons of the central nervous system (8). The availability of astrocytoma and neuroblastoma cells in culture affords an opportunity to study separately the glycogen metabolism in cells of neuronal and glial origin. The synthesis and breakdown of glycogen in cultures of C-6 astrocytoma cells and C-1300 neuroblastoma cells have been examined under normal growth conditions and in the presence of glucose 10 times greater than that in the usual medium. A cycle of reciprocal changes in the active forms of glycogen phosphorylase and glycogen synthase occurred when fresh medium was added to cells in the stationary phase of culture. These changes were accompanied by changes in the concentrations of intracellular glucose, glucose-6-P, UDP-glucose, and glycogen, but not of cyclic adenosine 3':5'-monophosphate. Furthermore, the administration of insulin increased the active form of glycogen synthase and decreased the amount of phosphorylase in the active form, without changes in cyclic AMP. The changes effected by insulin are more pronounced when glucose is added at the same time in the C-6 astrocytoma cells.

The effects of agents which did cause increases in cyclic AMP concentrations in the cells were also examined. Norepinephrine was effective in increasing the intracellular cyclic AMP concentrations in the astrocytoma cells. Isobutyl methylxanthine had a somewhat marginal effect on cyclic AMP, but did increase the active form of glycogen phosphorylase and diminish synthase a. In the neuroblastoma cells prostaglandin E1 and isobutyl methylxanthine caused an increase in intracellular cyclic AMP. In both cell lines, when cyclic AMP concentrations were elevated, phosphorylase was activated, synthase a was decreased, and glycogen content decreased.

MATERIALS AND METHODS

C-6 astrocytoma and C-1300 neuroblastoma (clone N-2a) cell lines were obtained from American Type Culture Collection (Rockville, Maryland 20852). The abbreviation used is: cyclic AMP, cyclic adenosine 3':5'-monophosphate.

"The abbreviation used is: cyclic AMP, cyclic adenosine 3':5'-monophosphate."

1Mersmann and Segal (9) have presented a persuasive argument to refer to the physiologically active form of glycogen synthase as the a form and the inactive form as the b form, in rough analogy to the phosphorylase system. Their suggestions have been adopted in this report.
The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 5.5 mM glucose, and 1 mM pyruvate (Grand Island Biological Co.) with 50 μg/ml of streptomycin and 38 units/ml of penicillin.

Prostaglandin E, a gift from J. Pike, Upjohn Co., Kalamazoo, Mich. 3-isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co., Milwaukee, Wis. UDP-[U-14C]glucose (210 mCi/mmol) was purchased from International Chemical and Nuclear Corp. All other reagents were purchased from Sigma Chemical Company, St. Louis, Mo. Enzymes used for the analysis of metabolites and of glycogen synthase and phosphorylase activity were purchased from Boehringer Mannheim Corp., New York.

Enzyme Assays—Glycogen phosphorylase was assayed at 25°C according to Lowry et al. (10). Glycogen synthase activity was measured either in a 2-step procedure in which UDP formation was measured fluorometrically (11), or by the incorporation of UDP-[U-14C]glucose into glycogen (12).

Metabolite Measurements—Glycogen, UDP-glucose, and glucose-6-P were measured with enzymic procedures according to Lowry and Passonneau (13). Glycogen was measured with amylo-1,4,1,6-glucosidase, hexokinase, and glucose-6-P dehydrogenase (14). Adenosine-3',5'-cyclic monophosphate was measured with a modified binding assay with the use of a commercial phosphodiesterase preparation (15). Protein was measured according to Lowry et al. (16).

Statistical analyses were made by Student's t test.

Preparation of Tissues—For the analysis of glycogen phosphorylase and glycogen synthase activities in the cells, the Petri dishes were rinsed three times with 2 ml of ice-cold 0.05 M Tris HCl, pH 7.5, containing 100 mM KF, 5 mM EDTA, and 0.5 mM dithiothreitol. Three rinses sufficed to remove all traces of the serum protein and glucose present in the standard medium. The dishes were then dropped into liquid nitrogen. The cells were then frozen in liquid nitrogen. The cells were then suspended in 1 ml of ice-cold 0.03 N HCl. An aliquot was removed for analysis of the other compounds. The analysis for UDP-glucose was destroyed in 0.03 N HC1 if the sample is heated or frozen. Recovery is complete, however, after 5 hours at 0°C.

The metabolite concentrations are expressed as nanomoles or picomoles per mg of protein, and enzyme velocities as nanomoles per mg of protein per hour. To facilitate comparison with other tissues the concentration of protein was determined to be near 10% for each cell line. (Average cell volumes were determined to be 2000 μm3 and 2700 μm3, and protein concentration 0.22 mg/cell and 0.28 mg/cell for the C-6 astrocytoma cells and C-1300 neuroblastoma cells respectively. A tissue density of 1 was assumed for calculation.)

Special Procedures—When the effect of various agents on the enzymes and metabolites was to be studied, the medium was removed and the cells were kept at 0°C. UDP-glucose is not stable in 0.03 N HC1 if the sample is heated or frozen. Recovery is complete, however, after 5 hours at 0°C.

The intracellular concentrations of glycogen and related metabolites were found to fluctuate depending on the amount of glucose in the medium (Table I, Fig. 1). Within 5 min after addition of fresh medium, the intracellular glucose concentration was 50-fold greater than that 2 hours after feeding. Glucose-6-P also rapidly increased in the cells to an even greater degree. UDP-glucose increased more slowly to a peak concentration as a function of time after feeding. C-6 cells were grown for 10 days as described under "Materials and Methods." The old medium was removed, and fresh medium containing serum was added. Medium samples were taken and the cells frozen at the times indicated. ○—●, medium glucose; ——, intracellular glycogen.

Results

The intracellular concentrations of glycogen and related metabolites were found to fluctuate depending on the amount of glucose in the medium (Table I, Fig. 1). Within 5 min after addition of fresh medium, the intracellular glucose concentration was 50-fold greater than that 2 hours after feeding. Glucose-6-P also rapidly increased in the cells to an even greater degree. UDP-glucose increased more slowly to a peak concentration as a function of time after feeding. C-6 cells were grown for 10 days as described under "Materials and Methods." The old medium was removed, and fresh medium containing serum was added. Medium samples were taken and the cells frozen at the times indicated. ○—●, medium glucose; ——, intracellular glycogen.

Concentrations of metabolites and cyclic AMP in C-6 astrocytoma cells at intervals after feeding

The cells were grown as described under "Materials and Methods." On the day of the experiment, the medium was removed, fresh medium with serum added, and the cells were frozen at the stated intervals as described under "Materials and Methods." The number of dishes analyzed at each interval is given in parentheses and the means ± S.E. are given when more than 2 dishes were used. The enzymes and metabolites were measured as described under "Materials and Methods."

Table I

| Time after feeding | Glucose | Glucose-6-P | UDP-glucose | Cyclic AMP |
|-------------------|---------|-------------|-------------|------------|
| 5 min (2) | 27.6 | 9.3 | | |
| 15 min (4) | 27.1 ± 1.0 | 5.1 ± 0.2 | 0.54 ± 0.06 | 18.0 ± 3.2 |
| 30 min (4) | 20.6 ± 1.0 | 3.7 ± 0.3 | 0.60 ± 0.11 | 15.6 ± 1.5 |
| 1 hr (10) | 14.1 ± 0.4 | 2.7 ± 0.2 | 1.12 ± 0.10 | 16.7 ± 1.7 |
| 2 hr (4) | 6.2 ± 1.0 | 0.94 ± 0.26 | 1.00 ± 0.19 | |
| 3 hr (6) | 6.1 ± 0.5 | 1.25 ± 0.08 | 1.87 ± 0.12 | 13.5 ± 2.2 |
| 5 hr (4) | 2.6 ± 1.0 | 0.70 ± 0.23 | 2.30 ± 0.06 | 19.4 ± 1.4 |
| 7 hr (8) | 2.0 ± 0.38 | 0.88 ± 0.10 | 2.21 ± 0.09 | 20.6 ± 1.4 |
| 9 hr (9) | 1.8 | 1.05 | 1.87 | 16.8 |
| 12 hr (2) | 0.58 | 0.02 | 0.86 | 15.0 |
| 24 hr (8) | 0.35 ± 0.1 | 0.06 ± 0.01 | 0.44 ± 0.10 | 19.1 ± 1.1 |

Concentrations of metabolites and cyclic AMP in C-6 astrocytoma cells at intervals after feeding

The cells were grown as described under "Materials and Methods." On the day of the experiment, the medium was removed, fresh medium with serum added, and the cells were frozen at the stated intervals as described under "Materials and Methods." The number of dishes analyzed at each interval is given in parentheses and the means ± S.E. are given when more than 2 dishes were used. The enzymes and metabolites were measured as described under "Materials and Methods."

Concentrations of metabolites and cyclic AMP in C-6 astrocytoma cells at intervals after feeding

The cells were grown as described under "Materials and Methods." On the day of the experiment, the medium was removed, fresh medium with serum added, and the cells were frozen at the stated intervals as described under "Materials and Methods." The number of dishes analyzed at each interval is given in parentheses and the means ± S.E. are given when more than 2 dishes were used. The enzymes and metabolites were measured as described under "Materials and Methods."
to decrease immediately after the addition of fresh medium and remained low for an hour. Subsequently, the percentage in the active form began to increase and at 24 hours was almost 60% of the total enzyme. Throughout the entire period there were no significant changes in cyclic AMP concentrations.

Not only did the percentage of synthase in the active form change, but the total activity varied (Table III). When the amount of enzyme in the a form is subtracted from the total activity, the amount of enzyme in the b form is relatively constant. The synthase a and b activities show striking activity, the amount of enzyme in the b form is relatively high glucose concentrations reflect inadequate re-

Because the concentration of extracellular glucose appeared to be critical, the effect of increased glucose in the medium was investigated. When medium glucose was 50 mM, higher concentrations of intracellular glucose were seen than with 5 mM glucose (Table III, cf. Table I). The glucose-6-P levels, however, were comparable in both experiments. The possibility that the high glucose concentrations reflect inadequate removal of the medium cannot be ruled out. However, the intracellular concentrations do not vary directly with the medium concentration, and the glucose may in fact be elevated. Such concentrations can be produced in neural tissue in diabetic animals (2, 20). The UDP-glucose concentrations are, if anything, decreased. The total phosphor-lyase activity is unchanged, while the percentage in the active form is decreased in the higher glucose medium and remains lower.

The amount of synthase in the active form is elevated up to 30 min and then decreased in the high glucose medium, both in percentage of total and amount of activity. In contrast to the normal medium the total synthase activity is markedly decreased after 3 and 7 hours in the high glucose medium. In medium with high glucose concentrations, the formation of an inactive species of synthase is apparently favored (see under “Discussion”).

Changes in the glucose concentration of the medium produced marked changes in the enzymes and metabolites in C-6 cells without any changes in cyclic AMP. Consequently, the effect of insulin, which facilitates the transport of glucose into certain cells, was examined. In experiments with liver, the effects of insulin in some cases have appeared to be dependent on the presence of glucose (21, 22), while other investigators have claimed that insulin effects were independent of glucose (23). When insulin was added in saline (0.9% NaCl solution) and medium glucose was 2100 μM, phosphorylase a was decreased and synthase a increased (Table IV). When the insulin was added in Dulbecco’s modified Eagle’s medium, which contains glucose, the medium glucose was increased to 2900 μM, and the changes in the amount of phosphorylase and synthase in the active forms were more marked. In addition, the increase glucose alone caused changes in both phosphorylase and synthase as compared to the saline controls (Table IV). The addition of insulin resulted in significant increases in intracellular glucose, glucose-6-P, and glycogen. All of these changes occurred without changes in the concentrations of cyclic AMP.

In order to determine the effects of altered concentrations of cyclic AMP, agents known or presumed to alter cyclic AMP concentrations in C-6 cells were tested. Norepinephrine has
The cells were grown as described under "Materials and Methods." On the 10th day in culture, the medium was removed and replaced with Dulbecco's modified Eagle's medium containing serum to which 10 mU insulin was added. After 20 min the medium was removed and fresh medium, with or without 50 mM glucose, was added. After 1 h the medium was removed and replaced with fresh medium containing 10 mU insulin. The medium was removed again and replaced with fresh medium containing 10 mU insulin and 50 mM glucose. The cells were frozen as described under "Materials and Methods." The number of dishes at each point is given in parentheses. The data are presented as in Table I. Units are: metabolite concentrations, nmol/mg of protein; cyclic AMP, pmol/mg of protein; enzyme activities, nmol of product/mg of protein/h.

| Time Point | Medium glucose, mM | Cell glucose | Glucose-6-P | UDP-glucose | Cyclic AMP | Phosphorylase a | Total phosphorylase | Percentage of phosphorylase a | Synthase a | Total synthase | Percentage of synthase a |
|------------|-------------------|-------------|-------------|-------------|-----------|----------------|---------------------|--------------------------|-----------|----------------|-------------------------|
| 15 Min     | 37.0 ± 0.9        | 73.4 ± 2.0  | 3.18 ± 0.11 | 0.72 ± 0.06 | 53.1 ± 1.2 | 5.54 ± 1.22 | 38.6 ± 1.3         | 555 ± 18                  | 162 ± 3   | 601 ± 22      | 27.4 ± 0.9              |
| 30 Min     | 41.7 ± 0.8        | 100.3 ± 5.5| 4.02 ± 0.21 | 0.23 ± 0.05 | 50.3 ± 9.6 | 8.77 ± 2.70 | 39.1 ± 1.4         | 538 ± 9                   | 80.5 ± 10.8 | 453 ± 32      | 17.1 ± 1.3              |
| 1 Hr       | 40.6 ± 1.0        | 104.0 ± 9.3| 3.32 ± 0.08 | 1.32 ± 0.03 | 53.6 ± 0.92| 4.72 ± 1.55 | 30.3 ± 0.9         | 558 ± 8                   | 25.6 ± 2.1 | 602 ± 22      | 5.1 ± 0.2              |
| 2 Hr       | 38.2 ± 1.4        | 89.7 ± 9.5  | 2.18 ± 0.11 | 1.72 ± 0.12 | 56.8 ± 2.1 | 4.05 ± 0.52 | 49.1 ± 2.8         | 558 ± 14                  | 5.5 ± 0.2   | 365 ± 14      | 1.5 ± 0.1              |
| 3 Hr       | 38.7 ± 1.1        | 78.9 ± 5.0  | 3.08 ± 0.08 | 2.22 ± 0.08 | 101 ± 1.8  | 7.03 ± 2.61 | 82.7 ± 7.3         | 599 ± 19                  | 1.1 ± 0.3   | 169 ± 4       | 0.9 ± 0.4              |
| 7 Hr       | 34.2 ± 1.5        | 58.7 ± 2.9  | 2.04 ± 0.14 | 2.22 ± 0.16 | 101 ± 4.2  | 10.40 ± 3.25 | 90.8 ± 3.7         | 616 ± 14                  | 1.1 ± 0.2   | 149 ± 6       | 0.7 ± 0.2              |

Table III
Concentrations of metabolites and cyclic AMP and activities of glycogen phosphorylase and glycogen synthase at intervals following feeding with 50 mM glucose

Table IV
Effect of insulin and/or glucose on glycogen phosphorylase, glycogen synthase, and related metabolites in C-6 astrocytoma cells in culture

The cells were grown as described under "Materials and Methods." On the 10th day of culture, the medium was removed, and 7 ml of fresh medium without serum added for 3 hours. Two milliliters of phosphate-buffered saline or Dulbecco's modified Eagle's medium were added to each dish. The cells were frozen as described under "Materials and Methods." The number of dishes at each point is given in parentheses. The data are presented as in Table I. Units are: metabolite concentrations, nmol/mg of protein; cyclic AMP, pmol/mg of protein; enzyme activities, nmol of product/mg of protein/h.

| Addition | PBS+ | PBS+ I' | DMEM+ | DMEM+ 1 |
|----------|------|--------|-------|---------|
| Medium glucose | 2173 ± 41 | 2134 ± 27 | 2937 ± 33 | 2845 ± 35 |
| Cell glucose | 4.21 ± 0.55 | 5.25 ± 0.35 | 5.25 ± 0.35 | 5.25 ± 0.35 |
| Glucose-6-P | 1.35 ± 0.16 | 2.15 ± 0.15 | 2.15 ± 0.15 | 2.15 ± 0.15 |
| UDP-glucose | 2.92 ± 0.36 | 2.93 ± 0.04 | 2.93 ± 0.04 | 2.93 ± 0.04 |
| Glycogen | 107 ± 5.2 | 121 ± 2.3 | 121 ± 2.3 | 121 ± 2.3 |
| Cyclic AMP | 11.9 ± 0.27 | 11.1 ± 0.48 | 11.1 ± 0.48 | 11.1 ± 0.48 |
| Percentage of phosphorylase a | 20.3 ± 0.6 | 14.3 ± 0.6 | 15.5 ± 0.7 | 6.81 ± 1.4 |
| Percentage of synthase a | 2.66 ± 0.2 | 4.80 ± 0.5 | 3.52 ± 0.06 | 7.24 ± 0.3 |

* PBS, phosphate-buffered saline.
* L, insulin.
* DMEM, Dulbecco's modified Eagle's medium.
* Insulin-treated samples different from controls; p < 0.001.
* Insulin-treated sample different from control; p < 0.01.
* Insulin-treated samples different from DBCM; p < 0.05.
* DMEM different from PBS; p < 0.01.

been shown to affect an increase in cyclic AMP concentration, phosphorylase a activity, and a decrease in glycogen concentration in C-6 cells (24, 25). These observations were confirmed in the present experiments (Table V). It was further observed that norepinephrine caused a significant decrease in intracellular glucose and the percentage of synthase in the active form. There were no changes in the concentrations of glucose-6-P or UDP-glucose in the cells, nor any effect on total enzyme activities (data not given).

Isobutyl methylxanthine, a phosphodiesterase inhibitor, and adenosine, a stimulator of adenylyl cyclase in brain slices, were evaluated for their effects on the enzymes and metabolites of the glycogen system. When 1 mM isobutyl methylxanthine was added to the cultures, there were marked increases in the fraction of phosphorylase in the active form and decreases in the active form of synthase at 30 and 45 min (Table VI). Glycogen was decreased to 60 and 50% of control values at 30 and 45 min, respectively. There were no consistent changes in cyclic AMP at either of the two time periods, although at other time intervals after feeding, isobutyl methylxanthine has caused a 2-fold elevation in cyclic AMP in the glioma cells (26).

No changes occurred in the concentrations of glucose, glucose-6-P, or UDP-glucose (data not given). Other experiments confirmed small but consistent changes in glycogen, phosphorylase a, and synthase a and no measurable changes in cyclic AMP. The data are not included since the control values for glycogen, phosphorylase a, and synthase a varied enough to make the standard errors very large. These variations are a result of the metabolic state of the cell as a function of time after feeding. The experimental times here are chosen so that a substantial fraction of phosphorylase is in the active form.
Glycogen Metabolism in Astrocytoma and Neuroblastoma Cells

Table V
Effect of norepinephrine on cyclic AMP, glycogen, and glucose concentrations and activities of glycogen phosphorylase and glycogen synthase in C-6 astrocytoma cells in culture

The cells were grown as described under "Materials and Methods." On the 10th day of culture, fresh medium without serum was added and 10 min later 0.1 mM norepinephrine was added. The cells were incubated for 20 or 35 additional min, the medium removed, and the cells frozen and prepared for analysis. The number of dishes in each group is shown in parentheses and values are given as the means ± S.E.

| Time after feeding | Time with norepinephrine | Glucose | Glycogen | Cyclic AMP | Percentage of phosphorylase a | Percentage of synthase a |
|--------------------|--------------------------|---------|----------|------------|-----------------------------|-------------------------|
| min                |                          | nmol/mg | pmol/mg  |            |                             |                         |
| 30 (5)             |                          | 22.4 ± 0.6 | 39.2 ± 1.5 | 6.3 ± 1.3 | 12.9 ± 0.6                  | 18.4 ± 0.4              |
| 30 (5)             |                          | 13.5 ± 0.9 | 61.4 ± 2.7 | 3.9 ± 1.3 | 12.5 ± 1.3                  | 13.3 ± 0.7              |
| 45 (3)             |                          | 19.1 ± 0.9 | 61.4 ± 2.7 | 3.9 ± 1.3 | 12.5 ± 1.3                  | 13.3 ± 0.7              |
| 45 (3)             |                          | 15.5 ± 0.1 | 39.0 ± 1.5 | 103 ± 3.1 | 44.5 ± 1.2                  | 55.1 ± 0.1              |

* Values different from controls; p < 0.001.
* Values different from controls; p < 0.01.
* Values different from controls; p < 0.02.

Table VI
Effect of isobutyl methylxanthine and adenosine on cyclic AMP and glycogen concentrations and activities of glycogen phosphorylase and glycogen synthase in C-6 astrocytoma cells in culture

The cells were grown as described under "Materials and Methods." On the 10th day of culture, fresh medium without serum was added and 10 min later 1 mM isobutyl methylxanthine + 0.25 mM adenosine was added and the cells incubated for an additional 20 or 35 min. The medium was removed and the cells frozen and prepared for analysis as described in the text. Values are the average of 2 dishes in each group. Units are: glycogen, nmol/mg of protein; cyclic AMP, pmol/mg of protein.

| Time after feeding | Addition | Glycogen | Cyclic AMP | Percentage of phosphorylase a | Percentage of synthase a |
|--------------------|----------|----------|------------|-------------------------------|-------------------------|
| min                |          | nmol/mg  | pmol/mg    |                               |                         |
| 30 min             | O        | 21.4     | 26.0       | 8.1                           | 13.9                    |
|                    | IBMX*    | 16.4     | 21.6       | 40.4                          | 6.7                     |
|                    | IBMX + adenosine | 16.6     | 19.2       | 29.8                          | 7.9                     |
| 45 min             | O        | 35.0     | 19.1       | 8.5                           | 11.0                    |
|                    | IBMX     | 18.0     | 30.5       | 55.1                          | 7.7                     |
|                    | IBMX + adenosine | 30.6     | 35.5       | 41.3                          | 6.6                     |

* IBMX, isobutyl methylxanthine.

Effect of isobutyl methylxanthine and adenosine on cyclic AMP and glycogen concentrations and activities of glycogen phosphorylase and glycogen synthase in C-6 astrocytoma cells in culture

The cells were grown as described under "Materials and Methods." On the 10th day of culture, fresh medium without serum was added and 10 min later 1 mM isobutyl methylxanthine + 0.25 mM adenosine was added and the cells incubated for an additional 20 or 35 min. The medium was removed and the cells frozen and prepared for analysis as described in the text. Values are the average of 2 dishes in each group. Units are: glycogen, nmol/mg of protein; cyclic AMP, pmol/mg of protein.

| Time after feeding | Medium glucose | Cell glucose | Glucose-6-P | UDP-glucose | Glycogen | Cyclic AMP |
|--------------------|----------------|--------------|-------------|-------------|----------|------------|
| min                |                |              |             |             |          |            |
| 10 min (2)         | 4998           | 34.8         | 2.37        | 1.25        | 2.13     | 25.7       |
| 20 min (2)         | 5014           | 31.8         | 2.99        | 1.38        | 2.86     | 25.5       |
| 30 min (2)         | 4732           | 32.6         | 3.37        | 1.46        | 10.7     | 35.9       |
| 1 hr (3)           | 4547           | 32.6         | 3.41        | 1.69        | 22.1     | 30.3       |
| 2 hr (2)           | 4233           | 24.4         | 2.57        | 1.76        | 42.8     | 23.0       |
| 3 hr (2)           | 3580           | 20.4         | 2.82        | 2.14        | 35.2     | 26.2       |
| 5 hr (2)           | 2945           | 10.2         | 1.78        | 2.36        | 18.8     | 37.4       |
| 7 hr (9)           | 896            | 9.60         | 0.90        | 2.97        | 16.4     | 31.0       |
| 9 hr (2)           | 267            | 2.37         | 1.49        | 1.69        | 8.7      | 20.2       |
| 12 hr (2)          | 20             | 0.64         | 0.26        | 1.07        | 2.5      | 23.8       |

* Values different from controls; p < 0.001.
* Values different from controls; p < 0.01.
* Values different from controls; p < 0.02.

The effect of insulin with and without added glucose on metabolite concentrations and the amount of phosphorylase and synthase in the active forms was investigated. At 3 and 5 hours after feeding, insulin was added to the medium in either saline or Dulbecco's modified Eagle's medium containing 5 mM glucose (Table IX). At 3 and 5 hours after feeding, insulin administration resulted in decreased phosphorylase a and increased synthase a. The effect on phosphorylase at 3 hours was significant when glucose was added with the insulin. Conversely, at 5 hours the effect of insulin on phosphorylase was seen only in the absence of added glucose. Synthase a increased in all cases except at 5 hours in the absence of glucose. These changes occurred without any significant changes in other metabolites related to glycogen metabolism (data not given) or cyclic AMP. Total enzyme activities were not affected.

The response of neuroblastoma cells to the phosphodiesterase inhibitor, isobutyl methylxanthine, and a stimulator of adenylate cyclase, prostaglandin E, (27), was investigated (Table X). The neuroblastoma cells showed a greater increase in cyclic AMP levels in the presence of isobutyl methylxanthine than the astrocytoma cells. There was an approximate 3-fold increase in cyclic AMP concentration which was accompanied by an increase in phosphorylase a activity and a decrease in synthase a activity. There were no changes in UDP-
**Glycogen Metabolism in Astrocytoma and Neuroblastoma Cells**

**Activities of glycogen phosphorylase and glycogen synthase in C-1300 neuroblastoma cells at intervals after feeding**

The cells were treated as in Table VII. Values for 2 to 5 dishes at each interval (in parentheses). Values are means ± S.E. when appropriate.

| Time after feeding | Phosphorylase | Synthase |
|--------------------|---------------|----------|
|                    | α             |          |          | α            |          |
|                    | nmol/mg of protein/hr | Total | Percentage of α | nmol/mg of protein/hr | Total | Percentage of α |
| 10 min (5)         | 69.6 ± 4.1    | 258 ± 15 | 27.0      | 25.1 ± 1.4    | 115 ± 5 | 21.8          |
| 20 min (5)         | 37.5 ± 3.5    | 310 ± 18 | 12.1      | 40.7 ± 2.5    | 145 ± 5 | 20.3          |
| 30 min (2)         | 27.0          | 230     | 11.4      | 28.6          | 116     | 24.6          |
| 1 hr (3)           | 30.6 ± 0.4    | 324 ± 40 | 10.4      | 35.2 ± 2.9    | 154 ± 0 | 21.5          |
| 2 hr (2)           | 47.0          | 248     | 19.0      | 18.5          | 136     | 13.6          |
| 3 hr (2)           | 48.9          | 232     | 21.0      | 10.2          | 112     | 9.1           |
| 5 hr (2)           | 53.2          | 233     | 22.8      | 10.5          | 87      | 12.1          |
| 7 hr (2)           | 61.4          | 234     | 26.2      | 13.0          | 88      | 14.7          |
| 9 hr (2)           | 64.2          | 219     | 30.9      | 22.4          | 92      | 24.2          |
| 12 hr (2)          | 141           | 217     | 65.0      | 18.4          | 81      | 22.7          |

**DISCUSSION**

The results indicate that glycogen metabolism in cells of neural origin in culture can be controlled in at least two ways, by the availability of a nutrient source, in this case glucose, and by alterations in the concentrations of cyclic AMP.

In both cell lines, glycogen increased for several hours after the medium was replaced with fresh medium containing 5 mM glucose. Initially, the amount of synthase in the active form was high; however, glycogen continued to increase even as the active enzyme was diminished (Tables I and II, Fig. 1). There appeared to be a maximum level of glycogen which is in some way regulated in the cell. The regulation may be due in part to changes which occur in metabolites and enzymes when the compounds are given separately to the cells. The increase in cyclic AMP concentration was more than additive and the changes in phosphorylase and synthase activity persisted. The increase in cyclic AMP concentration was more than additive and the changes in phosphorylase and synthase activity were accentuated. In addition, there was a substantial decrease in glycogen as well as in glucose and glucose-6-P.

**Effect of insulin on glycogen phosphorylase, glycogen synthase, and cyclic AMP in neuroblastoma cells in culture**

The cells were grown as described under “Materials and Methods.” The number of dishes is given in parentheses, and the values expressed as means ± S.E. when appropriate.

| Time after feeding | Addition | Medium glucose | Cyclic AMP | Percentage of phosphorylase α | Percentage of synthase α |
|--------------------|----------|----------------|------------|-------------------------------|--------------------------|
| 3                  | PBS (3)  | 2855 ± 75      | 22.0 ± 0.3 | 7.68 ± 0.5                   |                          |
|                    | PBS + I (3) | 2901 ± 31    | 16.8 ± 1.8 | 12.1 ± 0.3                   |                          |
|                    | DMEM (1) | 3248 ± 58     | 19.5 ± 0.5 | 8.24 ± 0.4                   |                          |
| 5                  | PBS (3)  | 2676 ± 74      | 15.0 ± 1.2 | 14.2 ± 0.4                   |                          |
|                    | PBS + I (3) | 2116 ± 96    | 22.3 ± 0.8 | 8.08 ± 0.7                   |                          |
|                    | DMEM (3) | 3272 ± 53     | 23.2 ± 0.9 | 6.60 ± 0.1                   |                          |
|                    | DMEM + I (3) | 3280 ± 68    | 22.0 ± 1.2 | 8.56 ± 0.3                   |                          |

- PBS, phosphate-buffered saline.
- I, insulin.
- Values different from controls; p < 0.01.
- Values different from control; p < 0.001.

**Glycogen Metabolism in Astrocytoma and Neuroblastoma Cells**

**TABLE VIII**

The cells were treated as in Table VII. Values are for 2 to 5 dishes at each interval (in parentheses). Values are means ± S.E. when appropriate.

| Hours after feeding | Addition | Medium glucose | Cyclic AMP | Percentage of phosphorylase α | Percentage of synthase α |
|--------------------|----------|----------------|------------|-------------------------------|--------------------------|
| 3                  | PBS (3)  | 2855 ± 75      | 22.0 ± 0.3 | 7.68 ± 0.5                   |                          |
|                    | PBS + I (3) | 2901 ± 31    | 16.8 ± 1.8 | 12.1 ± 0.3                   |                          |
|                    | DMEM (1) | 3248 ± 58     | 19.5 ± 0.5 | 8.24 ± 0.4                   |                          |
| 5                  | PBS (3)  | 2676 ± 74      | 15.0 ± 1.2 | 14.2 ± 0.4                   |                          |
|                    | PBS + I (3) | 2116 ± 96    | 22.3 ± 0.8 | 8.08 ± 0.7                   |                          |
|                    | DMEM (3) | 3272 ± 53     | 23.2 ± 0.9 | 6.60 ± 0.1                   |                          |
|                    | DMEM + I (3) | 3280 ± 68    | 22.0 ± 1.2 | 8.56 ± 0.3                   |                          |

- PBS, phosphate-buffered saline.
- I, insulin.
- Values different from control; p < 0.01.
- Values different from control; p < 0.02.

**TABLE IX**

The cells were treated as in Table VII. Values are for 2 to 5 dishes at each interval (in parentheses). Values are means ± S.E. when appropriate.

| Hours after feeding | Addition | Medium glucose | Cyclic AMP | Percentage of phosphorylase α | Percentage of synthase α |
|--------------------|----------|----------------|------------|-------------------------------|--------------------------|
| 3                  | PBS (3)  | 2855 ± 75      | 22.0 ± 0.3 | 7.68 ± 0.5                   |                          |
|                    | PBS + I (3) | 2901 ± 31    | 16.8 ± 1.8 | 12.1 ± 0.3                   |                          |
|                    | DMEM (1) | 3248 ± 58     | 19.5 ± 0.5 | 8.24 ± 0.4                   |                          |
| 5                  | PBS (3)  | 2676 ± 74      | 15.0 ± 1.2 | 14.2 ± 0.4                   |                          |
|                    | PBS + I (3) | 2116 ± 96    | 22.3 ± 0.8 | 8.08 ± 0.7                   |                          |
|                    | DMEM (3) | 3272 ± 53     | 23.2 ± 0.9 | 6.60 ± 0.1                   |                          |
|                    | DMEM + I (3) | 3280 ± 68    | 22.0 ± 1.2 | 8.56 ± 0.3                   |                          |

- PBS, phosphate-buffered saline.
- I, insulin.
- Values different from control; p < 0.01.
- Values different from control; p < 0.02.

Table VIII

Table IX
*PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.
* IBMX, isobutyl methylxanthine.

**TABLE X**

Effect of prostaglandin E<sub>2</sub> and/or isobutyl methylxanthine on glycogen phosphorylase, glycogen synthase, and related metabolites in C-1300 neuroblastoma cells in culture

The cells were grown for 10 days as described under "Materials and Methods." Fresh medium without serum was added and 5 μM prostaglandin E<sub>2</sub> and/or 1 mM isobutyl methylxanthine was added 30 min later and the cells incubated for an additional 20 min. The number of dishes is given in parentheses, and the values are means ± S.E. Units are: metabolite concentrations, nmol/mg of protein; cyclic AMP, pmol/mg of protein.

| Additive                      | Control (12) | IBMX<sup>*</sup> (6) | PGE<sub>2</sub><sup>*</sup> (6) | IBMX + PGE<sub>2</sub> (8) |
|------------------------------|--------------|----------------------|-------------------------------|----------------------------|
| Medium glucose, μM           | 4280 ± 74    | 4520 ± 150           | 4430 ± 286                    | 4170 ± 137                 |
| Cell glucose                 | 22.0 ± 0.3   | 18.8 ± 0.9<sup>a</sup>| 21.6 ± 0.9                    | 13.6 ± 0.6<sup>a</sup>     |
| Glucose-6-P                  | 2.57 ± 0.09  | 2.50 ± 0.08          | 2.72 ± 0.18                   | 1.54 ± 0.07<sup>a</sup>    |
| UDP-glucose                  | 1.82 ± 0.04  | 1.90 ± 0.04          | 2.03 ± 0.08<sup>a</sup>       | 1.95 ± 0.04<sup>a</sup>    |
| Glycogen                     | 13.6 ± 1.1   | 14.6 ± 1.0           | 15.2 ± 0.6                    | 8.0 ± 0.8<sup>a</sup>      |
| Cyclic AMP                   | 39 ± 8.7     | 117 ± 5.5<sup>d</sup> | 92 ± 8.7<sup>d</sup>          | 453 ± 47<sup>d</sup>       |
| Percentage of phosphorylase α| 11.8 ± 0.2   | 31.7 ± 1.5<sup>d</sup> | 24.7 ± 0.4<sup>d</sup>       | 41.0 ± 1.5<sup>d</sup>     |
| Percentage of synthase α     | 16.4 ± 0.4   | 13.4 ± 0.5<sup>d</sup> | 12.8 ± 0.2<sup>d</sup>        | 11.9 ± 0.5<sup>d</sup>     |

<sup>a</sup> Values different from control; p < 0.001.
<sup>d</sup> Values different from control; p < 0.05.

Glycogen synthase is not in operation. If there is inadequate or unavailable substrate for glycogen synthesis, the amount of active enzyme is not critical. The critical medium glucose level was about 2.5 mM in the astrocytoma cell, while in the neuroblastoma cells glycogen began to decrease when medium glucose was 4 mM. The rate of glucose transport into the cells cannot be the limiting factor, since the apparent K<sub>m</sub> values for transport in both cell lines are well below the medium glucose concentration, 1.7 mM glucose for C-6 cells, and 1 mM glucose for neuroblastoma cells (28).

The maximal rate of glycogen synthesis in the astrocytoma cells occurred between 5 and 15 min after feeding and was equivalent to 186 nmol/mg of protein/hour (Fig. 1). This is remarkably close to the synthase α activity 15 min after feeding, which is 172 nmol/mg of protein/hour (Table II). In the neuroblastoma cell line, the maximal rate of synthesis was 48 nmol/mg of protein/hour between 20 and 30 min after feeding (Table VII). The measured synthase α activity at 20 min after feeding was 41 nmol/mg of protein/hour. At other time periods, the correlation between the rate of glycogen accumulation and synthase α activity is similar. The conditions in the cell must be such that synthase α can operate at maximum velocity in both cell lines at these times.

It is of interest to note that with normal medium, total synthase activity in the C-6 cells decreases up to 5 hours after feeding, and this is due almost entirely to a decrease in synthase α (Table II, Fig. 2). After feeding with 50 mM glucose, there is a striking decrease in both synthase α and total synthase. In the C-1300 neuroblastoma cell line, there is also a decrease in synthase activity, although less pronounced than in the C-6 cells (Table VIII). The cause of the loss of total enzyme activity is not known. The enzyme could be degraded, but this seems unlikely to occur so rapidly, as the synthase of astrocytoma cells after 3 hours in 50 mM glucose is 28% of the initial amount (Table III). Furthermore, when neuroblastoma cells are refed, the synthase activity is increased 1.4-fold in 10 min (Table VIII). The cells appear to be viable and the phosphorylase activity remains constant. The loss of activity of synthase may be related to the phenomenon of inactive forms of glycogen synthase observed by others. There appears to be at least two main types of inactive enzyme. First, there are those forms which are reversibly inactivated during enzyme purification (3, 29-31) which can be restored by incubation with various compounds. Secondly, intermediate forms have been described which are formed during the conversion of the α to β form which are inactive (32, 33). This enzyme species is postulated to be more phosphorylated than the α form but less than the β form. In addition, the β form has been made inactive by incubation with ATP and Mg<sup>2+</sup> and is thought to be "extraphosphorylated" (33). It is attractive to consider that when the cells are in a medium with plenty of available nutrient and glycogen concentrations are high, a mechanism may exist that not only regulates the amount of enzyme in the active form, but can reduce the amount of total enzyme activity available without protein degradation. The inactive species may have extra phosphate groups, formed while energy stores are high. A further possibility is that a glycogen-enzyme inactive complex is formed. In the C-6 cells at least, the synthase activity is lowest when glycogen stores are highest, and the cells presumably are in a "saturated" state. The synthase-glycogen complex may become inactive when glycogen levels are very high, if all the enzyme is tightly bound.

The effects of insulin are similar in both cell lines, although the changes observed in metabolite levels in C-6 cells were not seen in the neuroblastoma cells. Furthermore, the changes were more marked in general when glucose was added with the insulin (Tables IV and IX). All of these effects occurred without alteration in the cyclic AMP concentration. In studies with perfused livers, Miller and Larner (23) associated insulin action with decreased cyclic AMP concentrations and decreased protein kinase activity. However, the decreased cyclic AMP was observed only if the livers were treated consecutively with glucagon and insulin, and not when insulin alone was used. It is possible that some subtle changes occur in a small pool of cyclic AMP in the cells (34); it seems more likely that the insulin effect is related to the metabolic effects demonstrated with glucose. Increases in synthase α activity following insulin administration have been observed by others in liver (35-38), diaphragm (39-42), skeletal (43) and heart muscle (44, 45), and adipose tissue (46).

In the C-6 cells, the mode of effect of isobutyl methylxanthine with or without adenosine on the phosphorylase and...
synthase activities and glycogen concentration is not clear. In one experiment cyclic AMP was not changed, and in a second, the concentration of cyclic AMP were about doubled (Table VI). In other experiments done at different time intervals after feeding, cyclic AMP was consistently elevated by this treatment (26). It seems likely that the enzyme changes do reflect an effect on the cyclic nucleotide. No changes were ever observed with adenosine alone.

The neuroblastoma cell line responded to prostaglandin E₁ and isobutyl methylxanthine with increased cyclic AMP concentrations, and concomitant changes in metabolites and the forms of phosphorylase and synthase (see under “Results” and Table X). All of the changes were more marked when both compounds were given, as would be expected since prostaglandin E₁ stimulates adenylate cyclase, and isobutyl methylxanthine inhibits phosphodiesterase.

In conclusion, there appears to be at least two ways of regulating glycogen metabolism in these cell lines. The active forms of synthase and phosphorylase vary with the glucose content of the medium and as a consequence, either form or degrade glycogen. When glucose is added to starved cells, glycogenolysis is favored. All of these events occur without discernible changes in cyclic AMP concentrations. However, if agents are used which increase the cyclic AMP in the cells, phosphorylase α is increased, synthase α is decreased, and glycogenolysis is favored.

REFERENCES

1. Coxon, R. V., Gordon-Smith, E. C., and Henderson, J. R. (1965) Biochem. J. 97, 776-781
2. Nelson, S. R., Schulz, D. W., Passonneau, J. V., and Lowry, O. H. (1968) J. Neurochem. 15, 1271-1279
3. Strong, R. H. C., and Bachelard, H. S. (1971) J. Neurochem. 18, 1067-1076
4. Goldberg, N. D., and O'Toole, A. G. (1969) J. Biol. Chem. 244, 3053-3061
5. Watanabe, H., and Passonneau, J. V. (1973) J. Neurochem. 20, 1543-1554
6. Nahorski, S. R., and Rogers, K. J. (1974) J. Neurochem. 23, 579-587
7. Guth, L., and Watson, P. K. (1969) Exp. Neurol. 22, 590-602
8. Passonneau, J. V., and Lowry, O. H. (1971) in Recent Advances in Quantitative Histology and Cytochemistry (Thibault, U. C., and Schmidt, U., eds) Vol. 3, pp. 198-209, Hans Huber, Bern
9. Mersmann, H. J., and Segal, H. L. (1967) Proc. Natl. Acad. Sci. U. S. A. 55, 1688-1695
10. Lowry, O. H., Schulz, D. W., and Passonneau, J. V. (1967) J. Biol. Chem. 242, 271-280
11. Passonneau, J. V., and Rotenberg, D. A. (1973) Anal. Biochem. 51, 528-541
12. Thomas, J. A., Schlender, K. K., and Larner, J. (1968) Anal. Biochem. 25, 486-490
13. Lowry, O. H., and Passonneau, J. V. (1972) A Flexible System of Enzymatic Analysis, pp. 174, 179, 217, Academic Press, New York
14. Passonneau, J. V., and Lauderdale, V. R. (1974) Anal. Biochem. 60, 405-412
15. Lust, W. D., Dye, E., Deaton, A. V., and Passonneau, J. V. (1976) Anal. Biochem., in press
16. Lowry, O. H., Rosebrugh, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
17. Lust, W. D., Passonneau, J. V., and Urites, S. K. (1975) Anal. Biochem. 68, 328-331
18. Danforth, W. H. (1965) J. Biol. Chem. 240, 588-593
19. Villar-Palasi, C. (1969) Arch. Biochem. Biophys. 166, 719-730
20. Passonneau, J. V., Brunner, E. A., Molstad, C., and Passonneau, R. (1971) J. Neurochem. 18, 2317-2328
21. Glinsmann, W. H., Pauk, G., and Hern, E. P. (1970) Biochem. Biophys. Res. Commun. 39, 774-782
22. DeWulf, H., and Hess, H. G. (1967) Eur. J. Biochem. 2, 50-56
23. Miller, T. B., Jr., and Larner, J. (1973) J. Biol. Chem. 248, 3483-3488
24. Opler, L. A., and Makman, M. H. (1972) Biochem. Biophys. Res. Commun. 46, 1140-1145
25. Browning, E. T., Schwartz, J. P., and Breckenridge, B. M. (1974) Mol. Pharmacol. 10, 162-174
26. Schwartz, J. P., and Passonneau, J. V. (1975) Fed. Proc. 34, 694
27. Gilman, A. G., and Nirenberg, M. (1971) Nature 234, 356-358
28. Lust, W. D., Schwartz, J. P., and Passonneau, J. V. (1975) Mol. Cell. Biochem. 8, 169-176
29. Steiner, D. F. (1961) Biochim. Biophys. Acta 54, 206-209
30. Haukuri, S., and Larner, J. (1964) Biochemistry 3, 1790-1788
31. Sanada, Y., and Segal, H. L. (1971) Biochem. Biophys. Res. Commun. 45, 1159-1168
32. Hadeckov, C. J., Esmann, V., and Rosell Pérez, M. (1966) Biochim. Biophys. Acta 130, 380-400
33. Morey, P., and Rosell-Perez, M. (1973) Rev. Espan. Fisiol. 29, 73-89
34. Soderling, T. R., and Park, C. R. (1974) in Advances in Cyclic Nucleotide Research (Greengard, P., and Robison, G. A., eds) Vol. 4, pp. 281-313, Raven Press, New York
35. Steiner, D. F., Rauda, V., and Williams, R. H. (1961) J. Biol. Chem. 236, 299-304
36. Kreutner, W., and Goldberg, N. D. (1967) Proc. Natl. Acad. Sci. U. S. A. 55, 1515-1519
37. Bishop, J. S., and Larner, J. (1967) J. Biol. Chem. 242, 1354-1356
38. Hostmark, A. T. (1970) Acta Physiol. Scand. 86, 246-255
39. Villar-Palasi, C., and Larner, J. (1961) Arch. Biochem. Biophys. 94, 436-442
40. Craig, J. W., and Larner, J. (1964) Nature 202, 971-973
41. Craig, J. W., Rall, T. W., and Larner, J. (1969) Biochim. Biophys. Acta 177, 213-219
42. Haukuri, S., and Larner, J. (1968) Biochim. Biophys. Acta 148, 660-672
43. Goldberg, N. D., Villar-Palasi, C., Sasko, H., and Larner, J. (1967) Biochim. Biophys. Acta 154, 464-464
44. Williams, B. J., and Mayer, S. E. (1966) Mol. Pharmacol. 2, 454-464
45. Hulshing, F., Nuttall, P. Q., Villar-Palasi, C., and Larner, J. (1969) Biochim. Biophys. Acta 177, 204-212
46. Jungas, R. L. (1966) Proc. Natl. Acad. Sci. U. S. A. 56, 757-763
Regulation of glycogen metabolism in astrocytoma and neuroblastoma cells in culture.

J V Passonneau and S K Crites

J. Biol. Chem. 1976, 251:2015-2022.

Access the most updated version of this article at http://www.jbc.org/content/251/7/2015

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/251/7/2015.full.html#ref-list-1