Effects of Adenosine 3′, 5′-Monophosphate and Adenosine 5′-Monophosphate on Glycogen Degradation and Synthesis in *Dictyostelium discoideum*

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Data are presented demonstrating that the presence in vivo of adenosine 3′, 5′-monophosphate (3′, 5′-AMP) causes a rapid depletion of glycogen storage material in the cellular slime mold. The effect of adenosine 5′-monophosphate (5′-AMP) is twofold, stimulating both glycogen degradation and synthesis. In pseudoplasmodia, cell-free extracts appear to contain at least two species of glycogen phosphorylase, one of which is severely inhibited by glucose-1-phosphate and another which is only partially inhibited by this hexose-phosphate. In some cases, 5′-AMP partially overcomes the inhibition by glucose-1-phosphate. Data presented here also indicate the existence of two forms of glycogen synthetase, the total activity of which does not change during 10 hr of differentiation from aggregation to culmination. During this period there is a quantitative conversion of glucose-6-phosphate-independent enzyme activity to glucose-6-phosphat-dependent activity. It is suggested that one effect of 3′, 5′-AMP is closely related to enzymatic processes involved in the rapid conversion of glycogen to cell wall material and other end products accumulating during sorocarp construction.

Differentiation in the cellular slime mold is initiated by starvation conditions and proceeds in the absence of external nutrients. The degradation of cellular proteins provides the major source of energy, and little gluconeogenesis occurs. Evidence suggests that the saccharide end products accumulating during the aging process are derived primarily from soluble glycogen present at the onset of starvation (5, 8, 9).

Studies from this laboratory have resulted in the construction of a quantitative kinetic model simulating a portion of the carbohydrate metabolism necessary to end-product accumulation (22, 23, 25). Analysis of this computer model predicted that the rate in vivo of glycogen synthesis and degradation (turnover) should be comparable to the rate of uridine diphospho-glucose (UDPG) synthesis at aggregation and that glycogen turnover should increase about threefold between the aggregation and culmination stages of differentiation. The model also predicted that, since glycogen phosphorylase, together with other glycogen-degrading enzymes, holds a critical kinetic position in this system, in vitro evidence might be found for an increase in the activity of these enzymes. These predictions have recently been substantiated (12, 16; R. A. Firtel and J. Bonner, Fed. Proc. 29: 669; T. H. D. Jones and B. E. Wright, Fed. Proc. 29: 670).

Concurrent with this emphasis on glycogen metabolism in *Dictyostelium discoideum*, work from J. T. Bonner’s laboratory resulted in the identification of acrasin as adenosine 3′, 5′-monophosphate (3′, 5′-AMP) (13), the chemotactic agent responsible for aggregation of myxamoebae into a multicellular pseudoplasmodium. In animal cells (21) and microorganisms (18, 20), 3′, 5′-AMP enhances glycogen degradation by stimulating phosphorylation of glycogen phosphorylase, thus converting this enzyme to its active form, and also by effecting the conversion of glycogen synthetase to a phosphorylated, inactive form. All of this information promoted an investigation into the effect of 3′, 5′-AMP on glycogen metabolism in vivo and in vitro in *D. discoideum*.

**MATERIALS AND METHODS**

Materials. 3′, 5′-AMP was obtained from Sigma Chemical Co.; dithiothreitol, adenosine 5′-monophosphate (5′-AMP), adenosine triphosphate (ATP), and oyster glycogen were obtained from Calbiochem.
Whatman diethylaminoethyl (DEAE) cellulose, DE 23, Balston, England, U-1 \(^{14}\)C-glucose was purchased from New England Nuclear Corp., and other chemicals were purchased from Fisher Scientific Co.

**Growth conditions and labeling of glycogen.** The amoebae of *D. discoideum* strain NC-4 were grown with *Escherichia coli* on nutrient agar surfaces, harvested, washed essentially free of bacteria, and spread on 2% agar plates containing phosphate buffer and ethylenediaminetetraacetate (EDTA); reference (15). The agar plates were incubated at 15 or 22 C.

When the desired stage of differentiation was obtained, the cells were harvested in cold 0.05 M potassium phosphate buffer, pH 6.4, and lightly homogenized with a Teflon-coated piston. A 3-ml amount of the cell suspension (packed cell volume, ca. 0.3 ml) was pipetted into a 50-ml Erlenmeyer flask to which 0.1 ml of 0.005 M \(^{14}\)C-glucose (2.8 \(\times\) 10\(^4\) counts per min per \(\mu\) mole) was added. The flask was shaken on a rotary shaker for 1 hr at room temperature (23 C). About ten identical flasks were needed for one in vivo experiment.

At the end of the incubation period, the cells were removed by centrifugation (2 min at 500 \(\times\) g) and washed twice in 20 ml of potassium phosphate buffer, pH 6.5. These cells were replated on plain agar and left at room temperature for 2 hr. Cells treated this way were used in the experiments in which unlabeled glucose was added as a chase.

\(^{14}\)C-glucose chase and isolation of labeled glycogen. Prelabeled cells were harvested in cold 0.05 M potassium phosphate buffer, pH 6.5, and homogenized lightly with a Teflon-coated piston. A 2-ml amount of the cell suspension (about 0.2 ml packed-cell volume) was added to a 50-ml Erlenmeyer flask containing a final volume of 3.0 ml: 0.05 M potassium phosphate buffer, pH 6.5; 0.16 \(\mu\) M \(^{14}\)C-glucose; and 1.0 mM 3',5'-AMP or 5'-AMP. Previous studies have established that cells treated in this manner differentiate normally thereafter and that measured reaction rates are comparable to those obtained with cells differentiating on agar (1-6).

The cells were exposed to \(^{14}\)C-glucose for several different chase periods. For each chase period three flasks were analyzed: one control, one containing 3',5'-AMP, and one with 5'-AMP. At the end of each chase period the contents of each flask were split into 0.5- and 2.5-ml portions. Each portion was mixed with an equal volume of cold 10% trichloroacetic acid and frozen at \(-20\) C overnight.

The larger portion was utilized to determine the specific radioactivity of glycogen. Cell debris was removed by centrifugation (1,000 \(\times\) g for 5 min) in a refrigerated portable centrifuge (International, model PR-2). The pellet was washed with 1 ml of 5% trichloroacetic acid, and after centrifugation the wash was combined with the first supernatant fluid. To the combined fluids 95% ethanol was added to a final concentration of 66%. The mixture was heated on a water bath to boiling and stored at \(-20\) C overnight. The precipitated glycogen was removed by centrifugation, dissolved in 0.5 ml of 1% LiCl, and reprecipitated by addition of 1 ml of 95% ethanol. This purification procedure was repeated three times. Finally, the glycogen precipitate was dissolved in 2 to 5 ml of distilled water. After removal of undissolved debris, an 0.5-ml sample was mixed with a liquid scintillation solution (Aquasol, New England Nuclear Corp., Boston, Mass.) and counted in a Beckman LS-200 scintillation counter (efficiency about 85\% with \(^{14}\)C). Another sample was used for the sugar determination.

The smaller portion obtained for each chase period was used for the determination of total radioactivity in the glycogen. Glycogen carrier (2 mg) was added to each portion, and the mixtures were treated as described above for the isolation of glycogen. After the purification procedure, the glycogen from each portion was dissolved in 0.3 to 0.5 ml of distilled water, and the whole sample was used for a determination of total radioactivity.

**Sugar analysis.** Glycogen content was determined by the method of Dubois et al. (10). Oyster glycogen was used as a standard.

**Packed cell volume.** Packed cell volume was determined, and a correction was made for intercellular water as previously described (3, 16).

**Preparation of enzyme extract.** At the desired stage of differentiation, the cells were harvested in cold 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.8, containing either 5 mM MgCl\(_2\) or 5 mM EDTA, and frozen for 3 to 16 hr. After thawing, cell debris was centrifuged at 23,000 \(\times\) g for 15 min. A 3-ml amount of this extract was dialyzed for 16 hr at 4 C against 1,000 ml of 0.01 M Tris buffer, pH 7.8, containing 2.5 mM MgCl\(_2\). Any precipitate formed during dialysis was centrifuged at 23,000 \(\times\) g for 15 min. The resulting supernatant liquid was used in the assay described below.

Alternatively, cells were harvested in 0.02 M Tris buffer, pH 7.8, containing 5 mM MgCl\(_2\) and Cemulsol NPT-12 (Melle-Bezons, 95 Bezons, France). Whole cells and cell debris were removed under conditions described above.

**In vitro glycogen degradation.** \(^{14}\)C-glycogen was prepared from culminating cells incubated in the presence of \(^{14}\)C-glycogen as described above. After the 60-min labeling period, 3 ml of 60% (w/v) KOH was added to each 3-ml cell suspension. The alkaline mixture was heated for 15 min on a boiling water bath. Isolation and purification were performed as above. The precipitated glycogen was further purified by washing two times with 95% ethanol and dissolving in 0.05 M 2-(N-morpholino)ethane sulfonic acid (MES)-KOH buffer, pH 6.5, from which it was precipitated by adding two volumes of 95% ethanol. Finally the radioactive glycogen was dissolved in the same buffer, and oyster glycogen was added to a final concentration of 0.5% (w/v), containing 8 \(\times\) 10\(^4\) to 10 \(\times\) 10\(^4\) counts per min per ml. The assay mixture contained: 0.1 ml of 0.5% glycogen in 0.05 M MES-KOH, pH 6.5; 0.05 ml of 0.1 M potassium phosphate buffer, pH 6.5; 0.025 ml of 0.05 M CaCl\(_2\); 0.001 ml of 0.25 M dithiothreitol; additives, as specified, in a total volume of 0.05 ml; and to 0.025 ml of enzyme extract. The final volume was 0.25 ml; the incubation temperature was 25 C.

The reaction was stopped by heating on a boiling water bath for 5 min, 0.5 ml of distilled water was added, and denatured protein was removed by centrifugation (1,000 \(\times\) g, 5 min). To separate glucose-1-phosphate from glucose, maltose, and partially degraded glycogen, a modification of a previously de-
scribed method (19) was employed. The supernatant liquid was quantitatively pipetted on to a microcolumn of 60 to 80 mg of DEAE cellulose contained in a disposable Pasteur pipette. The sample in a volume of about 0.75 ml filtered through the column under gravity in 5 to 10 min. The column was washed with 0.5 ml of twice-distilled water. Absorbed material was eluted directly into a liquid scintillation vial by using 0.1 M potassium phosphate buffer, pH 6.9 (1 ml followed by 0.5 ml).

Radioactivity was determined as above. A blank containing no enzyme was carried through the same procedure. This value was subtracted from those obtained in the experimental samples.

Alternatively, we precipitated partially degraded glycogen in the presence of 2 mg of glycogen carrier by using ethanol.

Degradation products soluble in 66% ethanol were separated as described above, and the radioactivity of nonabsorbed products, such as glucose and maltose, were determined separately.

In vitro glycogen synthesis. Total synthesizing capacity of enzyme extracts was determined in the presence of 1 mM glucose-6-phosphate (I + D) activity. The glucose-6-phosphate-independent activity was also determined (I-activity). Subtraction of I-activity from (I + D) activity was taken as a measure of glucose-6-phosphate-dependent (D)-activity alone.

The assay mixture in a total volume of 0.2 ml consisted of: 0.1 ml of 0.5% glycogen in 0.02 M Tris buffer, pH 7.8; 0.005 ml of 14C-UDPG (1.4 × 105 cpm/min), 0.02 ml of enzyme extract, 0.07 ml; and Tris buffer and additives.

The reaction was initiated by the addition of enzyme and incubated at 25 C for the time periods indicated. The reaction was stopped by the addition of 0.2 ml of 10% trichloroacetic acid. A precipitate was removed by centrifugation, and the pellet was washed successively with 0.2 ml of 0.5% glycogen and 0.2 ml of 1% LiCl. To the combined supernatant fluids, two volumes of 95% ethanol were added. The precipitated glycogen was redissolved in 1% LiCl and further purified, and the radioactivity was determined as described above.

Protein content. The protein content was determined by the method of Lowry et al. as described by Bailey (1). Bovine serum albumin (Sigma Chemical Co.) was used as a standard of reference.

RESULTS

In vivo degradation of glycogen. Cells at the pseudoplasmodium stage of differentiation which had been exposed to 14C-glucose were suspended in liquid cultures containing 14C-glucose at a final concentration of 0.16 mM. In the first 10 to 15 min after exposing cells to a glucose chase, we found that prelabeled material was rapidly incorporated into glycogen, resulting in a transient increase in total radioactivity, amount, and specific radioactivity of the glycogen pool. All experiments indicated that this high-specific-radioactivity glycogen was rapidly degraded in the next 30 min.

Figure 1 shows the total radioactivity of glycogen at seven time intervals and the specific radioactivity at five time intervals. From these data of total and specific radioactivity we are able to calculate the actual content of glycogen at each time interval.

The results for total radioactivity of the glycogen pool indicates that a more rapid degradation of glycogen occurs in the presence of 3',5'-AMP as compared to 5'-AMP and the control. The loss of total radioactivity in the presence of 5'-AMP is comparable to that of control; however, the specific radioactivity of glycogen in the presence of 5'-AMP being lower than that of control, 5'-AMP must also stimulate glycogen degradation, as the probability of losing radioactivity from the pool is proportional to the specific radioactivity of the pool.

From the calculated data for total glycogen content at each time interval, it appears that, after an initial loss, the glycogen level is again increased. In the presence of 5'-AMP, this resynthesis of glycogen proceeds over a 60-min period at a rate twice that of control, with 3',5'-AMP giving an intermediate value.

When the same experiment was performed over a 30-min period in the absence of a glucose chase, the total and specific radioactivity of glycogen in the absence of added nucleotides stayed
constant. In the presence of 3',5'-AMP or 5'-AMP, total radioactivity decreased to a level 45% of that of control. During the first 15 min, no loss of radioactivity was observed in the presence of 5'-AMP as compared with a 30% loss in presence of 3',5'-AMP. In the latter case, the amount of glycogen remaining, as calculated from total and specific radioactivities, was 75% of that of the control and of the sample containing 5'-AMP. Thus, qualitatively, the nucleotides showed comparable effects on glycogen degradation in the presence or absence of a glucose chase.

Experiments with no glucose chase were also performed with cells not previously exposed to 1^4C-glucose. Other conditions were as described above.

Cells from two stages of differentiation were incubated in liquid cultures, and total glycogen content was determined as glucose equivalents per milliliter of packed cell volume (Fig. 2).

The effect of 5'-AMP on the glycogen content is strikingly different from that of 3',5'-AMP. At culmination, in the presence of 5'-AMP, an increase in glycogen content is clearly observed. This must be attributable to a relatively greater stimulation of glycogen synthesis as compared to degradation, rather than to an inhibition of degradation only, as 5'-AMP stimulates glycogen degradation (Fig. 1). At the pseudoplasmodium stage of differentiation, 5'-AMP has relatively little effect on the glycogen content as compared to the control. Cells at the pseudoplasmodium and early culmination stages of differentiation rapidly deplete their glycogen supply when suspended in liquid medium containing 3',5'-AMP.

Studies in vitro of glycogen degradation and synthesis were undertaken to investigate specific reactions which could be responsible for the observed effects in vivo of 5'-AMP and 3',5'-AMP.

In vitro degradation of 1^4C-glycogen. Glycogen phosphorylase and amylase have been isolated from cell-free extracts from all stages of differentiation (12; P. A. Rosness, 154th National Meeting of the American Chemical Society, 1967), and glycogen phosphorylase has been partially purified and characterized (12). In two independent investigations of glycogen phosphorylase from D. discoideum, no stimulation by 3'5'-AMP or 5'-AMP was found (12; R. A. Firtel and J. Bonner, Fed. Proc. 29:669; T. H. D. Jones and B. E. Wright, Fed. Proc. 29:670). The assay method used [glucose-1-phosphate production coupled enzymatically to reduced nicotinamide adenine dinucleotide phosphate (NADPH) production] does not allow a determination of end-product inhibition by G-1-P. An assay was therefore constructed for the determination of total glycogen-degrading capacity in crude enzyme extracts by using 1^4C-glycogen as substrate. The 1^4C-glycogen was isolated from the culmination stage after the cells were exposed to 1^4C-glucose for 60 min in liquid cultures. The composition of the assay system is described above.

The charged degradation products from 1^4C-glycogen, e.g., glucose-1-phosphate, were absorbed on dry DEAE-cellulose powder. Noncharged products, e.g., glucose and maltose, were not absorbed, and radioactivity of such compounds could therefore be determined separately after partially degraded glycogen was precipitated with ethanol. Glucose-1-phosphate was eluted from the DEAE cellulose by using 0.1 M potassium phosphate buffer, pH 6.9. This method, based on radioactivity determinations, recovered 99% of a commercial preparation of glucose-1-phosphate, and 97% of a glucose sample was not absorbed. DEAE-cellulose paper chromatography of the glucose preparation gave the same result; about 3% of the total radioactivity stayed at the origin.

The isolated and purified 1^4C-glycogen used in the in vitro experiments contained about 10%
charged impurities; the remaining 90% did not absorb to the DEAE cellulose under our assay conditions. Commercially available glycogen phosphorylase (twice crystallized from rabbit muscle; Sigma Chemical Co.) incubated with the assay mixture gave a linear release of radioactive material with time until about 25% of the original radioactivity was released.

After incubation of ¹⁴C-glycogen with a diazoylized enzyme preparation from the pseudoplasmodium stage of differentiation, the enzymatic product subsequently absorbed to the DEAE cellulose was identified as glucose-1-phosphate by using the previously mentioned spectrophotometric assay. When inorganic phosphate in the assay mixture was replaced by MES (0.05 M, pH 6.5), no glucose-1-phosphate was formed, whereas the amount of nonabsorbed material (resulting from amylase activity) was 92% of that in the presence of phosphate.

With this assay, it was possible to confirm a previous finding (12) that nucleotide sugars such as adenosine diphosphoglucose (ADPG) and guanosine diphosphoglucose (GDPG) inhibited glycogen phosphorylase completely at a final concentration of 1 mM. In the presence of 1 mM ADPG, complete inhibition occurred after a time lag of 5 to 10 min, indicating that the glycogen preparation was contaminated with a minor fraction (less than 5% based on radioactivity) that was degraded to a charged product absorbing to DEAE cellulose. A similar but shorter lag of inhibition by ADPG was found by using the spectrophotometric assay with oyster glycogen as substrate. Therefore, the isolated ¹⁴C-glycogen was incubated under the assay conditions for 5 min with enzyme extract, heated for 10 min at 100°C, and reisolated by the methods described above. The resulting glycogen was used for investigations of glycogen phosphorylase activity.

One-day-old diazoylized enzyme extract from the pseudoplasmodium stage of differentiation was used in the time study shown in Fig. 3. Rates of phosphorolysis were determined without and with the addition of 5'-AMP, 5'-AMP + glucose-1-phosphate, and glucose-1-phosphate. The glucose-1-phosphate formed was absorbed and eluted from the DEAE cellulose as described previously. A nonenzymatic control was included, and the radioactivity eluted from the cellulose in this sample was subtracted as background. This value was 200 counts/min in this experiment. As shown, 5'-AMP stimulates glycogen phosphorylase activity about 33% over control. Addition of glucose-1-phosphate to the assay mixture inhibits the reaction to a level 25% of that of the control. Glycogen phosphorylase activity assayed in the presence of glucose-1-phosphate and 5'-AMP is twice the rate determined in the presence of glucose-1-phosphate alone. Total phosphorylase activity was usually taken as that measured in the presence of 5'-AMP. It should be noted that the counting error gives a large standard deviation for the lower experimental values. Qualitatively, however, these results have been observed several times.

Occasionally, freshly prepared extracts after dialysis from both the pseudoplasmodium and culmination stages of development had no phosphorolysis activity in the presence of 1 mM glucose-1-phosphate but were fully active when 5'-AMP was added together with glucose-1-phosphate to the assay mixture. After storage at 4°C for 1 day, phosphorylase activity increased, and the enzyme activity was only partially inhibited by glucose-1-phosphate. The increase in total activity measured in the presence of 5'-AMP was comparable to, and thus could be attributed to, the increased activity in the presence of glucose-1-phosphate. Maximum increase was achieved after 2 days of storage.
In animal cells, phosphorylase b is converted to phosphorylase a in the presence of magnesium ions, ATP, and phosphorylase b kinase. This reaction is stimulated by 3',5'-AMP. Because of the effect of ATP, 3',5'-AMP, and ions on this conversion, it was of interest to carry out comparable investigations on the apparent conversion of a glucose-1-phosphate--inhibited to a glucose-1-phosphate--insensitive form of the enzyme. Thus, two 0.5-ml samples of enzyme extract (pseudoplasmodium) were incubated with 4 mM CaCl₂, 2.5 mM MgCl₂, and 1 mM dithiothreitol, and adjusted to pH 7.2 by using 0.05 mM MES, pH 6.5. To one of the samples, ATP and 3',5'-AMP were added to a final concentration of 1 mM. Both mixtures were incubated at 4°C for 3 days and then dialyzed overnight against 0.01 M Tris, pH 7.8, and 2.5 mM MgCl₂.

Before preincubation, activities assayed in the presence of 5'-AMP (total phosphorylase activity) and glucose-1-phosphate, respectively, were 910 and 357 counts per min per ml. After incubation, the control had the following activities (in counts per minute per milliliter): 792 in the presence of 5'-AMP and 408 in the presence of glucose-1-phosphate, compared to 780 and 656 for the enzyme extract incubated in the presence of ATP and 3',5'-AMP, respectively. The fraction of glycogen phosphorylase activity not inhibited by glucose-1-phosphate was increased by about 60% compared to the control preincubated in the absence of ATP and 3',5'-AMP.

In vitro glycogen synthesis. Previous studies from this laboratory indicated that the synthesis of soluble glycogen from UDPG is stimulated by glucose-6-phosphate and that this requirement varies with the enzyme preparation and temperature of assay (24). These data prompted us to study this requirement more thoroughly in the present investigation, as studies using enzyme preparations from animal cells have shown that a glucose-6-phosphate stimulation is related to the conversion of a nonphosphorylated (I) to a phosphorylated (D) form of the enzyme. The phosphorylated form of glycogen synthetase is dependent on glucose-6-phosphate for activity. As in the case of glycogen phosphorylase, this conversion requires magnesium ions, ATP, and a specific kinase and is stimulated by the presence of 3',5'-AMP. To avoid differential enzyme inactivation as a function of the stage of differentiation and to measure possible changes in the relative I- and D-activities as rapidly as possible, crude extracts were used in preference to the washed, 100,000 × g pellet fraction previously employed (24). Under these assay conditions, several crude enzyme preparations were 90 to 95% active in the absence of added glucose-6-phosphate. Thus it was assumed that I-activity was not significantly stimulated by glucose-6-phosphate and that D-activity could be calculated simply by subtracting I-activity from the total activity in the presence of glucose-6-phosphate.

As previous studies indicated that glycogen is rapidly degraded during the accumulation of cell wall material, we anticipated that, at this stage of differentiation, the synthetase would be converted to a less active form of the enzyme. Therefore (I + D) activity and I-activity were determined at several stages of differentiation prior to sorocarp construction. Cells were lysed in the presence of Cemulsol NPT12, a nonionic detergent, and 5 mM MgCl₂ (Table 1).

The I-activity decreased from the amoeba stage to culmination, whereas D-activity increased. Total (I + D) activity was constant from aggregation to culmination, suggesting a quantitative conversion of I-activity to D-activity. Although total (I + D) activity varied between amoeba and aggregation, D-activity stayed constant and was equal to the activity found in enzyme extracts prepared by freezing and thawing cells in the presence of 5 mM EDTA.

Cells at the aggregation stage of development were also treated for 30 min under in vivo conditions in the presence of 0.16 mM glucose with no addition or with either 5'-AMP or 3',5'-AMP. The cells were washed and lysed in the presence of detergent as above. Compared to an unincubated control, no change in total (I + D) or I-activity was observed in the absence of nucleotides. In the presence of 3',5'-AMP, total and I-activity decreased (about 50%); D-activity was the same as in the controls. The addition of 5'-

Table 1. Specific activity of glycogen synthetase during development of Dictyostelium discoideum

<table>
<thead>
<tr>
<th>Stage of differentiation</th>
<th>¹⁴C-uridine diphosphoglucone (amoles per min per mg of protein)</th>
</tr>
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<tbody>
<tr>
<td>I + D</td>
<td>I</td>
</tr>
<tr>
<td>Amoeba (2 hr)</td>
<td>2.7</td>
</tr>
<tr>
<td>Aggregation (8 hr)</td>
<td>1.45</td>
</tr>
<tr>
<td>Early pseudoplasmodium (11 hr)</td>
<td>1.37</td>
</tr>
<tr>
<td>Early culmination (17 hr)</td>
<td>1.37</td>
</tr>
<tr>
<td>Culmination (19 hr)</td>
<td>1.34</td>
</tr>
</tbody>
</table>

* At each stage of differentiation the cells were harvested in 0.02 M tris(hydroxymethyl)aminomethane, pH 7.8, containing 5 mM MgCl₂ and 0.15% Cemulsol NPT-12. Total glycogen synthetase activity (T + D) and glucose-6-phosphate--independent (I) activity were determined as described in Materials and Methods. Glucose-6-phosphate--dependent (D) activity was calculated by subtracting I from (T + D).
AMP resulted in no change in D-activity, but I-activity decreased to an intermediate value of the control and 3',5'-AMP.

A pseudoplasmodium enzyme extract was preincubated at 30 C for different time periods, and total (I + D) plus I-activity was assayed after each time interval (Fig. 4). The I-activity was almost completely destroyed after 60 min at 30 C, whereas the D-activity did not change. D-activity appears to be remarkably stable under a variety of conditions at the early stages of differentiation. Thus, various lines of evidence indicate the presence of two different species of glycogen synthetase.

DISCUSSION

The in vivo experiments presented here result in higher-specific-radioactivity glycogen and examine changes over shorter time intervals than had been observed previously (24). It was hoped that the sensitivity of the assay would thus be increased, allowing the detection of possible effects of 3',5'-AMP and 5'-AMP on glycogen metabolism. One by-product of using these experimental conditions is the observation that the addition of a glucose chase to prelabeled cells results in a transient increase in the specific radioactivity of glycogen. An explanation for this result could be that 14C-oligosaccharides in the maltose series are rapidly incorporated into glycogen and that the increase in specific radioactivity may also be enhanced by inhibition of degradation of glycogen by glucose-1-phosphate formed from added glucose. A rapid incorporation of oligosaccharides into high-molecular-weight glycogen has been observed earlier in another system (3). Apparently this newly synthesized glycogen produced in the first period of the chase is very rapidly turned over until a new steady-state level is reached and a lower rate of turnover is established (16). The transient effect of enhanced glucose levels on the size of the glycogen pool is consistent with an assumption of the kinetic model, i.e., that the accumulation of glucose, hexose phosphates and UDPG normally occurring during differentiation will result in an increase in the rate of glycogen synthesis.

Changes in total radioactivity with time in vitro are taken as a measure of glycogen degradation, and a change of specific radioactivity is assumed to reflect glycogen turnover. The degrading capacity of glycogen in the cells probably consists of at least two different classes of enzymes, the amylases and glycogen phosphorylase. Traditionally, glycogen phosphorylase is considered to be central to the regulation of glycogen degradation; in D. discoideum, however, amylase activity may play a significant role. We do not exclude the possibility that 3',5'-AMP, for instance, increases the amount of amylase activity per cell. Preliminary evidence indicates an increase in amylase specific activity observed in cell-free extracts prepared from cells exposed to 3',5'-AMP; concomitantly, no increase of glycogen phosphorylase activity was found.

Storage of phosphorylase at 4 C resulted in a characteristic increase of total activity assayed in the presence of 5'-AMP, and the appearance of phosphorylase activity not inhibited by glucose-1-phosphate was found. This process is accelerated by preincubation of the enzyme extract at 25 C.

When enzyme extracts from the pseudoplasmodium stage of differentiation were used, 5'-AMP always stimulated the activity of freshly prepared glycogen phosphorylase. When assayed in the presence of 10 mM EDTA instead of 5 mM CaCl2, a lesser stimulation was observed.

The reproducible effect of 5'-AMP on glycogen phosphorylase appears to be a decrease of end-product inhibition, not excluding the possibility that, analogous to phosphorylase b in animal cells (21) and some microorganisms (18, 20), some enzyme preparations are dependent on 5'-AMP for activity. Crude enzyme preparations from the pseudoplasmidium-to-culmination stages of development have been observed to be stimulated up to twofold by the presence of 5'-AMP in the assay mixture.

During the course of differentiation, glycogen synthetase activity independent of glucose-
6-phosphate decreases, whereas activity dependent on glucose-6-phosphate increases. The conversion of the independent to the dependent form has been shown to be stimulated by 3',5'-AMP in other systems (18, 20, 21). In D. discoideum, the addition of 3',5'-AMP to cells at aggregation decreased I-activity 50%, but no concomitant increase of D-activity was found. During the first 8 hr of differentiation, until aggregation, D-activity stays constant while I-activity varies with the conditions and method of isolation of the synthetase. At aggregation, it is also possible to demonstrate an increase of glucose-6-phosphate-independent activity by freezing and thawing in the presence of EDTA. Hizukuri and Larner (11) have reported that conversion of D-activity to I-activity goes through an inactive form and that the activation of the inactive form is faster than the direct conversion of D-activity to I-activity. These observations suggest that in this period of development (i.e., in the first 8 hr) a different mechanism of regulation exists.

The rate of differentiation and aging in D. discoideum is stimulated by 3',5'-AMP, 5'-AMP, and other mononucleotides (6, 7, 14). Based on observations recorded here, these stimulating effects may be related to changes in the rate of glycogen degradation and synthesis, which occur during differentiation (16). Effects on glycogen degradation are of particular interest mechanistically, in view of the critical kinetic position held by glycogen-degrading enzymes in this system (22, 23, 25). The data presented indicate that both glycogen phosphorylase and synthetase exist in two forms, the interconversion of which involves 3',5'-AMP.

5'-AMP may stimulate glycogen degradation by overcoming the glucose-1-phosphate inhibition of glycogen phosphorylase; in turn, the effect would stimulate glycogen synthesis through enhanced levels of glucose-1-phosphate and UDPG. In the case of 3',5'-AMP, degradation could be stimulated in part through the formation of 5'-AMP, but another effect must also be operative, as 3',5'-AMP is always more effective than 5'-AMP in stimulating glycogen degradation in the intact cell. Another mechanism is through the effect of 3',5'-AMP in the conversion of glycogen phosphorylase to a more active form. Furthermore, the conversion of a glucose-6-phosphate-independent to a glucose-6-phosphate-dependent form of glycogen synthetase occurs during differentiation, a transformation known to be stimulated by 3',5'-AMP. This conversion will result in decreased synthetase activity, as glucose-6-phosphate falls to undetectable levels during sorocarp construction. These combined effects, together with the stimulation of cell wall construction by 3',5'-AMP (6), may be crucial to the cessation of glycogen synthesis and its conversion to the end products of differentiation.

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LITERATURE CITED


