Glycogen Synthetase and the Control of Glycogen Synthesis in the Cellular Slime Mould *Dictyostelium discoideum* During the Growth (Myxamoebal) Phase

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1. Myxamoebae of the cellular slime mould *Dictyostelium discoideum* Ax-2 that are grown in axenic medium containing 86 mM-glucose have seven times the glycogen content of the same myxamoebae grown in the same medium but lacking added carbohydrate. 2. During the transition from the exponential to the stationary phase of growth in axenic medium containing glucose myxamoebae preferentially synthesize glycogen and can have as much as three times the glycogen content during the stationary phase as they have during the exponential phase of growth. 3. The rate of glycogen degradation by myxamoebae is, under all conditions of growth, small compared with the rate of glycogen accumulation and the changes in glycogen content thus reflect altered rates of glycogen synthesis. 4. There is no correlation between the rate of glycogen synthesis by myxamoebae and the glycogen synthetase content of the myxamoebae. 5. The activity of glycogen synthetase of *D. discoideum* is inhibited by a physiological concentration of ATP and this inhibition is overcome by glucose 6-phosphate. Both effects are especially marked at physiological concentrations of UDP-glucose. 6. The rate of glycogen accumulation by myxamoebae growing exponentially in axenic media can be satisfactorily accounted for in terms of the known intracellular concentrations of glucose 6-phosphate, UDP-glucose and glycogen synthetase. The rate-limiting factors controlling glycogen synthesis by the myxamoebae are apparently the substrate (UDP-glucose) and effector (glucose 6-phosphate and ATP) concentrations rather than the amount of the enzyme.

There are two distinct and conflicting models of differentiation in *Dictyostelium discoideum*. Sussman & Sussman (1969) have proposed that differentiation is controlled by a series of transcriptional and translational events ordered both in space and time and manifesting themselves as a series of changes in enzymic activities the concerted action of which results in the observed morphogenesis. Wright *et al.* (1968), by contrast, have argued that changes in enzyme content are irrelevant to the control of differentiation and that the observed changes in metabolic fluxes are caused by changes in the concentration of metabolites, as these concentrations are usually lower than the K_m values for the enzymes concerned. It is clear that, contrary to the report of Wright & Dahlberg (1968), the changes in enzyme activity reported by Sussman and his co-workers are genuine (Newell & Sussman, 1969; Edmundson & Ashworth, 1972). However, the observation that such changes occur does not prove that at all times during differentiation the rate-limiting step controlling a metabolic reaction is the concentration of enzyme catalysing it. The isolation of an axenic strain of *D. discoideum* that synthesizes variable amounts of

enzymes during growth (Ashworth & Quance, 1972) and that can successfully complete the standard morphogenetic sequence with a variety of enzyme assemblies (Quance & Ashworth, 1972) suggests that controls may exist at levels other than, or in addition to, those that are known to occur at transcription and translation.

In the present paper we discuss the factors that regulate the glycogen content of myxamoebae of *D. discoideum* Ax-2 during growth (Ashworth & Watts, 1970). We present evidence that it is the activity, and not the concentration, of glycogen synthetase that regulates the rate of glycogen synthesis of *D. discoideum* Ax-2.

Materials and Methods

Materials

ATP, NAD⁺, NADP⁺, glucose 6-phosphate, UDP-glucose, phosphoenolpyruvate, rabbit muscle glycogen, pyruvate kinase and UDP-glucose dehydrogenase were obtained from Sigma (London) Ltd., London W.5, U.K. Dowex 1 (X8; AG; Cl⁻ form) and Dowex 50 (X8; AG; H⁺ form) were obtained

from Bio-Rad Laboratories, St. Albans, Herts., U.K. Dowex 1 (formate form) was prepared from Dowex 1 (Cl⁻ form). *NN*-Diocetyl-methylamine was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. All other chemicals were of the highest purity available and were obtained from either BDH Chemicals Ltd., Poole, Dorset, U.K., or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K. [U-¹⁴C]Glucose and UDP-[¹⁴C]glucose were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Growth and harvesting of myxamoebae. The strains of *D. discoideum* used were the wild-type strain *D. discoideum* NC-4, and the axenic strain *D. discoideum* Ax-2 (Watts & Ashworth, 1970). Myxamoebae of strains NC-4 and Ax-2 were grown on agar plates in association with *Aerobacter aerogenes* (Sussman, 1966). They were harvested at the first sign of disappearance of the bacterial 'lawn' and were washed free of bacteria. Myxamoebae of strain Ax-2 were also grown in axenic media in the absence and presence of glucose, as described by Watts & Ashworth (1970). Cell densities were determined by counting with a haemocytometer.

Preparation of cell-free extracts. Washed myxamoebae were resuspended in 0.1 M-tris-chloride (pH 7.5)–0.0015 M-EDTA–0.0025 M-dithiothreitol. Sufficient buffer was added to give a cellular concentration of about 10⁸ myxamoebae/ml. Cells were disrupted with a Branson Sonifier for 1 min. The sonic treatment was limited to 15 s bursts, with the use of intermittent cooling to maintain the temperature of the suspension below 4°C. The resulting suspension was centrifuged at 5000g for 10 min to remove cell debris and unbroken cells. In some experiments the myxamoebae were disrupted by freezing and thawing a suspension. Cell suspensions were rapidly frozen at –40°C and then thawed under cold running water. This process was repeated and the resulting suspension was centrifuged at 5000g for 10 min to remove cell debris and unbroken cells. The supernatant fractions were assayed for glycogen synthetase activity and for glycogen content. In some experiments the 5000g supernatants were fractionated by centrifugation at 100000g for 1.5 h. The resulting pellet fraction was resuspended in the tris-chloride buffer and both the pellet and the 100000g supernatant were assayed for glycogen synthetase activity.

Measurement of protein and glycogen. The protein content of the cell-free extracts was determined by the method of Lowry *et al.* (1951). The glycogen content of myxamoebae and cell-free extracts was determined as alkali-soluble, alcohol-precipitable, anthrone-positive material (Cooper & Kornberg, 1967).

Determination of the rate of glycogen degradation. Cells were grown in axenic media containing 390 µCi of [U-¹⁴C]glucose/l. After growth to the appropriate cell density, cells were harvested and washed free of media. The cells were resuspended in fresh axenic media, containing 86 mM-glucose, at the same cell density that had been achieved during growth, and growth was allowed to continue. Samples were removed at 10 min intervals, the cell density was determined and the cellular glycogen was isolated. The glycogen was then dissolved in 1 ml of water and added to 10 ml of Triton–toluene scintillation fluid (Fox, 1968) and counted for radioactivity in a Tracerlab liquid scintillation spectrometer at 62% efficiency.

Determination of glycogen synthetase activity. Glycogen synthetase was determined by measuring the transfer of [U-¹⁴C]glucose from UDP-[U-¹⁴C]glucose to glycogen by a modification of the method of Piras *et al.* (1968). Incubation mixtures contained 7.5 µmol of tris-chloride, pH 7.5, 1.25 µmol of UDP-[U-¹⁴C]glucose (37 nCi/µmol), 1 mg of rabbit muscle glycogen and 0.2–0.5 mg of protein (extract) in a final volume of 0.125 ml and were incubated for 15 min at 30°C. Reactions were terminated by the addition of 1.5 ml of 0.1 M-ammonium acetate in 66% ethanol. The reaction tubes were immediately placed in a boiling-water bath for 15 s and then transferred to an ice bath for 15 min. The precipitated glycogen was washed once with 1.5 ml of 66% ethanol, and then resuspended in 1.5 ml of 66% ethanol. The glycogen was filtered through a 2.5 cm Whatman no. 1 filter paper disc, and the filter disc was washed with 5 ml of 66% ethanol and dried under an i.r. lamp. The dried disc was added to 10 ml of scintillation fluid [5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-(5-phenyloxazole-2-yl)benzene per l of toluene] and counted at approx. 50% efficiency in a Tracerlab liquid scintillation spectrometer.

Glycogen synthetase activity was also measured by determining the glycogen-dependent formation of UDP from UDP-glucose. The reaction mixtures contained 30 µmol of tris-chloride buffer, pH 7.5, 5 µmol of UDP-glucose, 4 mg of rabbit muscle glycogen and 0.2–0.5 mg of protein (extract) in a final volume of 0.5 ml. Control reactions contained no glycogen. The reaction mixtures were incubated at 30°C and the reaction was stopped after 15 min by placing the tubes in a boiling-water bath for 3 min. UDP formation was assayed by adding phosphoenolpyruvate to the UDP, in the presence of pyruvate kinase; the pyruvate produced was determined colorimetrically after the addition of 2,4-dinitrophenyl hydrazine (Leloir & Goldemberg, 1960).

In both determinations the reaction rates were linear with time and with the amount of added protein (extract).

Extraction and determination of glucose 6-phosphate

and UDP-glucose. Axenically grown myxamoebae of strain Ax-2, in exponential phase (approx. 10^9 total cells), were harvested by centrifugation in the 8×50 ml rotor of the M.S.E. 18 centrifuge. The rotor was rapidly accelerated to 2000g and then immediately decelerated. The culture medium was quickly decanted and the cell pellet was resuspended in 0.8 ml of 5% HClO_4 . The suspension was then sonicated for 30s. The whole process took less than 3 min and over 90% of the cells were collected by the rapid centrifugation technique.

The sonicated suspension was stored at 0°C for 30 min to allow complete extraction of metabolites, and was then centrifuged at 15000g for 30 min. The supernatant liquid was decanted and adjusted to approximately pH 7.0 by the addition of 30% KOH. The precipitated KClO_4 was removed by centrifugation and the supernatant was assayed directly for glucose 6-phosphate. Samples (0.5 ml) of the neutralized supernatant were also applied to columns (7 cm $\times$ 0.5 cm) of Dowex 1 (formate form). The column was washed first with 5 ml of 0.2M-formic acid and then with 5 ml of 0.2M-formic acid con-

taining 0.2M-ammonium formate. UDP-glucose was then eluted with 5 ml of 0.2M-formic acid containing 1.0M-ammonium formate. The eluate was applied directly to a column (26 cm $\times$ 1.5 cm) of Dowex 50 (H^+ form) to remove NH_4^+ . The UDP-glucose was eluted from the column with 40 ml of water and the solution was freeze-dried. The freeze-dried residue was redissolved in 0.4 ml of water and samples (0.1 ml) were assayed for UDP-glucose.

Assay mixtures for glucose 6-phosphate contained 20 μmol of triethanolamine chloride, pH 7.5, 160 nmol of NADP^+ , 10 μmol of MgCl_2 and 12.5 μg of glucose 6-phosphate dehydrogenase in a total volume of 1 ml. The assay reaction was initiated by the addition of glucose 6-phosphate dehydrogenase and the increase in fluorescence was determined with an Eppendorf fluorimeter. After completion of the reaction 1.25 μg of glucose 6-phosphate dehydrogenase and 15 nmol of glucose 6-phosphate were added and the increase in fluorescence was measured to provide an internal standard. The glucose 6-phosphate dehydrogenase did not contribute to the recorded fluorescence, and therefore a background subtraction was unnecessary. Assay mixtures for UDP-glucose contained 10 μmol of tris-chloride buffer, pH 8.5, 320 nmol of NAD^+ and 100 μg of UDP-glucose dehydrogenase in a total volume of 1 ml. The reaction was initiated by the addition of UDP-glucose dehydrogenase and the increase in fluorescence was determined. After completion of the reaction 100 μg of UDP-glucose dehydrogenase and 6.25 nmol of UDP-glucose were added to obtain an internal standard determination. The UDP-glucose dehydrogenase contributed to the recorded fluorescence; this contribution was subtracted from the increase in fluorescence caused by the reaction.

Results

Glycogen content of myxamoebae

It was shown (Ashworth & Watts, 1970) that the alkali-soluble, alcohol-precipitable, polysaccharide of axenically grown *D. discoideum* was glycogen, and that inclusion of glucose in the axenic medium increased the concentration of this cellular glycogen. Therefore myxamoebae of *D. discoideum* Ax-2 were grown both in axenic medium and in axenic medium containing 86 mM-glucose and samples of the culture were removed periodically during growth, and the glycogen content of the cells was determined. The glycogen content of cells grown in media containing 86 mM-glucose was about sevenfold that of cells grown in the absence of glucose (Fig. 1, see Ashworth & Watts, 1970). The cellular glycogen content remained constant during exponential growth in either medium (Fig. 1). However, in the medium containing glucose, there was an increase in

Fig. 1. Cellular glycogen concentrations at various times during growth

Myxamoebae of strain Ax-2 were grown axenically and samples were removed at the indicated times. Cell density was determined by counting cell number with a haemocytometer; ($\circ$), cells grown in the absence of glucose; (Δ), cells grown in the presence of 86 mM-glucose. Cellular glycogen content of cells grown in the absence of glucose ($\bullet$), and in the presence of 86 mM-glucose ($\blacktriangle$), was determined as described in the Materials and Methods section.

the cellular glycogen content when the growth rate began to decrease at the end of exponential growth. The glycogen content reached a maximum in the stationary phase, but further incubation of the cells eventually resulted in a decrease in the glycogen concentration. There were no corresponding changes in the glycogen content of the cells in media that did not contain added glucose (Fig. 1). The doubling time of myxamoebae in the axenic medium that did not contain glucose was 9h and in the axenic medium containing 86mM-glucose it was 8h. This compares with a doubling time of about 4h for strain Ax-2 grown in association with *A. aerogenes* (J. M. Ashworth, unpublished work) and about 3h for strain NC-4 grown in association with *A. aerogenes* (Sussman, 1966).

Glycogen metabolism during growth of myxamoebae

Glycogen degradation. The sevenfold difference in the glycogen content of cells growing exponentially in the presence of glucose compared with that of cells growing in the absence of glucose (Fig. 1) might be caused either by different rates of glycogen synthesis or different rates of glycogen degradation. The rate of glycogen degradation of myxamoebae, growing under different conditions, was therefore determined. When cells were transferred to fresh medium, to determine the rate of degradation of glycogen, the growth rate of the myxamoebae was normal or faster than normal (Fig. 2) suggesting that such cells were completely viable. The $t_{\frac{1}{2}}$ for glycogen degradation of cells grown in the presence of glucose was 36.5h and that for cells grown in the absence of glucose was 19.25h

(Fig. 2). These rates of glycogen degradation are very low relative to the rates of glycogen accumulation (Fig. 1), and if the rates of glycogen synthesis were identical the $t_{\frac{1}{2}}$ for glycogen degradation of myxamoebae grown in the absence of glucose would need to be approx. 40min to account for the sevenfold differences in glycogen accumulation.

The rates of degradation so determined may be lower than the true rates because of the decrease in specific activity of the glycogen during the course of experiments of the type shown in Fig. 2. This decrease (Fig. 2) is very small, however, and we have neglected it.

Glycogen synthesis. Since the rates of accumulation and degradation of glycogen *in vivo* are known (Figs. 1 and 2), the rates of glycogen synthesis can be determined. For myxamoebae grown in the presence of glucose, the rate of accumulation of glycogen, calculated from the glycogen content during the exponential phase, was $4.5 \mu\text{g}$ of glucose equivalents/min per 10^8 cells and the rate of glycogen degradation was $0.6 \mu\text{g}$ of glucose equivalents/min per 10^8 cells. Thus the rate of glycogen synthesis was $5.1 \mu\text{g}$ of glucose equivalents/min per 10^8 cells. Similarly, for myxamoebae grown in the absence of glucose, the rate of glycogen accumulation was $0.6 \mu\text{g}$ of glucose equivalents/min per 10^8 cells, and the rate of degradation was $0.174 \mu\text{g}$ of glucose equivalents/min per 10^8 cells; hence the rate of glycogen synthesis was $0.774 \mu\text{g}$ of glucose equivalents/min per 10^8 cells. Thus the difference in glycogen content of the myxamoebae grown in the presence and absence of glucose are caused by considerable differences in the rates of glycogen synthesis.

Fig. 2. Determination of rates of glycogen degradation of myxamoebae

Myxamoebae grown in medium containing $[U-^{14}\text{C}]$ glucose, either in the absence ($\circ$, $\bullet$) or presence (Δ , $\blacktriangle$) of 86mM-glucose, were harvested, washed and resuspended at the same concentration in fresh media containing 86mM-glucose at zero time. Samples of the culture media were removed at the indicated times and the cell density ($\circ$, Δ) and the radioactivity in the glycogen ($\bullet$, $\blacktriangle$) were determined.

Further, the increase in glycogen content of the glucose-grown myxamoebae as they emerge from the exponential phase (Fig. 1) cannot be accounted for solely by the decreased rate of cell division, and the increase is also too great to be accounted for by a decreased rate of glycogen degradation. The $t_{\frac{1}{2}}$ of glycogen degradation of myxamoebae in late log phase (10^7 myxamoebae/ml) was found to be 23 h and for myxamoebae in stationary phase (2×10^7 myxamoebae/ml) it was 16.5 h (the $t_{\frac{1}{2}}$ of glycogen degradation during the exponential phase was 36.5 h), demonstrating faster rather than slower degradation of glycogen as myxamoebae go into the stationary phase. Because the rate of glycogen synthesis probably increases continually as cells grow out of exponential phase there are insufficient results to determine the increased rate accurately. However, on average, during the transition from the exponential phase to the stationary phase the increase in rate of glycogen synthesis is approximately two- to three-fold.

Thus, under these different growth conditions, rates of glycogen synthesis *in vivo* vary considerably and a study of the enzyme glycogen synthetase was therefore undertaken.

Glycogen synthetase

Extraction and stability of the glycogen synthetase. Disruption of cells either by controlled sonication or by freeze-thaw treatment yielded extracts with similar glycogen synthetase activities (Table 1), whereas the yields after continuous sonication or Triton X-100 treatment were lower. Activity was found only in the cell-free extract (5000g supernatant).

Centrifugation at 100000g for 1.5 h yielded a pellet and a supernatant fraction that both contained glycogen synthetase activity, although the pellet had a consistently higher specific activity. However, the specific activity of the pellet varied from preparation to preparation (Table 1), and routine assays were, therefore, performed on the 5000g supernatant preparation.

The activity of the glycogen synthetase rapidly decreased after cell disruption. The inclusion of 1.5 mM-dithiothreitol in the extraction buffer stabilized the activity to a certain extent, but no detectable activity remained after storage of the extract at 22°C for 2 h, 4°C for 24 h, -20°C for 3 days or -65°C for 7 days. However, there was no decrease in the activity of extracts stored at -65°C for 24 h. The 100000g pellet and 100000g supernatant fractions were equally unstable. The glycogen synthetase activity of extracts prepared in 0.1 M-tris-chloride (pH 8.5)-0.025 M-EDTA, as described by Wright & Dahlberg (1967), was consistently lower, somewhat variable and equally unstable.

Stoichiometry of the glycogen synthetase. The rate of transfer of glucose from UDP-glucose to glycogen was equivalent to the rate of glycogen-dependent formation of UDP (Table 2). This stoichiometry existed for fresh and stored extracts of strain Ax-2, and for extracts of the wild-type strain NC-4.

Comparison of the rates of glycogen synthesis with the activities of the glycogen synthetase

The glycogen synthetase activity of cells grown in the presence of glucose was only slightly higher than

Table 1. Preparation of cell-free extracts for glycogen synthetase assays

Myxamoebae of strain Ax-2 were grown axenically on medium that contained 86 mM-glucose and were harvested when growth reached a cell density of 2×10^6 – 3×10^6 cells/ml. The extraction procedure is described in the Materials and Methods section. If the cell suspension was sonicated continuously for 3 min then less than 50% of the activity remained. A similar loss resulted from preparation of cell-free extracts by addition of Triton X-100. n.d., Not detectable.

Extraction method	Glycogen synthetase		
	Specific activity (nmol/min per mg of protein)	Total activity (nmol/min)	% of initial activity
Freeze and thaw treatment			
5000g supernatant	20.0	300	100
5000g pellet		n.d.	0
100000g supernatant	19.0	196	65
100000g pellet	31.4	135	45
Controlled sonication			
5000g supernatant	21.4	635	100
5000g pellet		n.d.	0
100000g supernatant	17.4	356	56
100000g pellet	48.2	280	44

Table 2. *Stoichiometry of glycogen synthetase reaction*

Myxamoebae of strain Ax-2 were grown axenically on medium containing 86mM-glucose to a cell density of 1×10^6 cells/ml. Strain NC-4 was grown in association with *A. aerogenes*. Cell-free extracts were prepared by controlled sonication, as described in the Materials and Methods section. Assays were done as described in the Materials and Methods section, except that, where indicated, 10mM-glucose 6-phosphate was included in the reaction mixture.

Strain	Extract	Glycogen synthetase activity	
		[U- ¹⁴ C]Glucose incorporation into glycogen (nmol/min per mg of protein)	UDP formation (nmol/min per mg of protein)
Ax-2	5000g supernatant*	16.4	15.4
Ax-2	5000g supernatant* + 10mM-glucose 6-phosphate	15.8	16.7
Ax-2	Stored 5000g supernatant†	9.5	7.8
NC-4	5000g supernatant*	5.8	7.8

* Extracts assayed immediately after preparation.

† Extract stored at -20°C for 20h.

that for cells grown in the absence of glucose, despite the considerable difference in the rates of glycogen synthesis under these conditions (Table 3). Further, the rates of glycogen synthesis *in vivo* were considerably lower than the maximum synthesizing capacity of the enzyme (Table 3). There was no change in the glycogen synthetase activity of cells grown in axenic media during the transition from the exponential to the stationary phase, despite the previously discussed two- to three-fold increase in the rate of glycogen synthesis. Although glycogen accumulated faster in the Ax-2 and NC-4 strains grown in association with *A. aerogenes* than in Ax-2 grown axenically in the absence of glucose, the glycogen synthetase activities were lower in the former cells. It is clear from these results that there was no correlation between the activity of the glycogen synthetase *in vitro* and the rate of glycogen synthesis *in vivo*.

Regulation of glycogen synthetase

UDP-glucose and glucose 6-phosphate concentrations. Because the activity of the glycogen synthetase *in vitro* was considerably greater than the rates of glycogen synthesis *in vivo* (Table 3), the rate of glycogen synthesis might be regulated by variations in the concentrations of UDP-glucose substrate, and the well-established effector of glycogen synthetase, glucose 6-phosphate. In fact, it was found that the inclusion of 86mM-glucose in the axenic growth medium resulted in a sixfold increase in the concentration of glucose 6-phosphate and a three- to four-fold increase in the concentration of UDP-glucose. As 10^9 cells have an internal volume of approx.

0.1ml the intracellular concentrations of UDP-glucose are therefore in the range 100–330 μM and the concentrations of glucose 6-phosphate are in the range 230 μM –1.5mM (assuming no intracellular compartmentation).

Effect of glucose 6-phosphate. Wright & Dahlberg (1967) reported that the glycogen synthetase of *D. discoideum* was markedly stimulated by glucose 6-phosphate. However, in the present study, glucose 6-phosphate, at a variety of concentrations, failed to stimulate the glycogen synthetase activity of extracts (Fig. 3; Table 2). The reason for these discrepancies is not clear, but it is possible that the stimulatory effect of glucose 6-phosphate is only exhibited after the partial purification of the enzyme described by Wright & Dahlberg (1967).

Effects of adenine nucleotides and glucose 6-phosphate. The glycogen synthetase of yeast is active in the absence of glucose 6-phosphate (Rothman & Cabib, 1967a). Nevertheless, the finding that physiological concentrations of adenine nucleotides markedly inhibit yeast glycogen synthetase activity, and that this inhibition is reversed by glucose 6-phosphate, has emphasized the importance of glucose 6-phosphate as a regulator of this enzyme (Rothman & Cabib, 1967b).

The glycogen synthetase of *D. discoideum* was markedly inhibited by ATP at low concentrations of the UDP-glucose substrate (Fig. 3). The other adenine nucleotides, ADP and AMP, also inhibit glycogen synthetase activity, and the concentrations of adenine nucleotides that are inhibitory are within the physiological concentration range (Jones, 1970). The inhibition of glycogen synthetase by ATP was reversed by the inclusion of glucose 6-phosphate in

Table 3. Comparison of the rates of glycogen synthesis *in vivo* with the activity of the glycogen synthetase

Myxamoebae were grown to the requisite cell density, as indicated below. Glycogen was determined as alkali-soluble, alcohol-precipitable, anthrone-positive material. The rate of glycogen accumulation was determined from the glycogen content and the generation time. The rate of glycogen degradation was determined from the glycogen content and the glycogen degradation results of Fig. 2. The rate of glycogen synthesis was determined as the sum of the rates of degradation and accumulation. Results are expressed as the means $\pm$ S.E.M. and the number of determinations are shown in parentheses.

Strain	Growth conditions	Cell density (myxamoebae/ml)	Glycogen content (mg of glucose equivalents/10 ⁸ myxamoebae)	Rate of glycogen accumulation (μ g of glucose equivalents/min per 10 ⁸ myxamoebae)	Rate of glycogen degradation (μ g of glucose equivalents/min per 10 ⁸ myxamoebae)	Rate of glycogen synthesis (nmol of glucose equivalents/min per mg of protein)	Glycogen synthetase activity (nmol of glucose equivalents/min per mg of protein)
Ax-2	Axenic medium	$3 \times 10^5 - 15 \times 10^5$	0.29 ± 0.02 (9)	0.54	0.17	0.4	15.0 ± 0.6 (8)
Ax-2		6×10^6	0.30 ± 0.02 (6)	—	—	—	13.4 ± 1.5 (6)
Ax-2	Axenic medium + 86 mM-glucose	$5.5 \times 10^5 - 40 \times 10^5$	2.15 ± 0.12 (6)	4.50	0.60	2.83	17.6 ± 0.5 (30)
Ax-2		8.5×10^6	4.80 (1)	approx. 9-13.5	approx. 2.0	—	19.4 (1)
Ax-2		1.5×10^7	5.60 ± 0.5 (4)	—	—	—	18.3 ± 1.1 (4)
Ax-2	Bacteria	—	0.38 ± 0.02 (3)	approx. 1.6	—	—	9.6 ± 0.6 (2)
NC-4			0.36 ± 0.08 (2)	approx. 2.0	—	—	6.1 ± 0.7 (4)

reaction mixtures (Fig. 3). Although the relationship between the concentrations of UDP-glucose, glucose 6-phosphate and the adenine nucleotides and the activity of the glycogen synthetase is complex, the results presented here suggest that the increased concentrations of UDP-glucose and glucose 6-phosphate in myxamoebae grown in the presence of glucose (Table 4) cause the increased rate of glycogen synthesis. Moreover, for myxamoebae grown in the presence of glucose, the activity of the glycogen synthetase at the physiological UDP-glucose concentration, 326 μ M (Table 4), can be calculated to be 1.7 nmol of glucose equivalents/min per mg of protein, assuming that the inhibitory effects of the adenine nucleotides are reversed by the elevated glucose 6-phosphate concentration. This value agrees closely with the rate of glycogen synthesis *in vivo*, which is 2.83 nmol of glucose equivalents/min per mg of protein (Table 3).

Discussion

The rate of biosynthesis of glycogen is thought to be controlled by allosteric regulation of the first unique enzymic step. Thus those organisms that use ADP-glucose as the glucosyl donor, such as bacteria and plants, possess an ADP-glucose pyrophosphorylase with activity that is sensitive to a variety of metabolic effectors (Preiss, 1969); in contrast, those organisms, such as mammals, yeast and *D. discoideum*, that use UDP-glucose as the glucosyl donor have an UDP-glucose pyrophosphorylase activity that is insensitive to such metabolites (Edmundson & Ashworth, 1972) and instead possess a glycogen synthetase activity that is subject to allosteric regulation. In such organisms the glycogen synthetase reaction is the rate-limiting step in glycogen synthesis (Rothman & Cabib, 1967*b*). The rate of degradation of glycogen in myxamoebae of *D. discoideum* is low relative to the rate of its accumulation (Table 3). Thus the rate of glycogen accumulation of a myxamoeba growing exponentially is a reasonably accurate measure of the integrated flux of carbon through the glycogen synthetase reaction. Glycogen synthetase can be assayed accurately and is sufficiently stable to enable kinetic studies of it to be undertaken. Further, sensitive assays exist for UDP-glucose and glucose 6-phosphate. Thus it is possible to monitor simultaneously changes in flux, enzymic content and substrate and effector concentrations for this reaction and hence to evaluate those factors that might regulate glycogen synthesis. Few other metabolic pathways are so convenient for such studies, and previous work (Quance & Ashworth, 1972) has suggested that this knowledge is essential if the factors controlling cell differentiation are to be understood.

The increases in the rate of glycogen synthesis

Fig. 3. Effect of glucose 6-phosphate and ATP on glycogen synthetase activity at various concentrations of the UDP-glucose substrate

Myxamoebae were grown axenically to 2×10^6 cells/ml and harvested. Extracts were prepared by freeze and thaw treatment as described in the Materials and Methods section. In the experiment shown in the inset myxamoebae were grown in the same way as before but extracts were prepared by controlled sonication, as described in the Materials and Methods section. Reaction mixtures (○) contained $15 \mu\text{mol}$ of tris-chloride, pH 7.5, 2 mg of rabbit muscle glycogen, 0.18 mg of protein from the 100000g pellet extract (or in the experiment shown in the inset: 0.37 mg of protein from 5000g supernatant extract), UDP-[U- ^{14}C]glucose (37 mCi/mol) as indicated, and water to a final volume of 0.25 ml. Also, reaction mixtures (●) contained $1.25 \mu\text{mol}$ of glucose 6-phosphate, reaction mixtures (△) contained $4 \mu\text{mol}$ of ATP, and reaction mixtures (▲) contained $1.25 \mu\text{mol}$ of glucose 6-phosphate and $4 \mu\text{mol}$ of ATP.

observed when myxamoebae were grown in the presence of glucose, and when myxamoebae grown in glucose entered the stationary phase of growth, might, *a priori*, be caused by an increase in the rate of synthesis of glycogen or a decrease in the rate of its degradation. However, as shown in Table 3, although the rates of degradation of glycogen change with changing growth conditions, they are always low compared with the rates of accumulation. Further, the changes that do occur cannot account for the changes in glycogen accumulation, because they are in the opposite sense, i.e. during the transition from exponential to stationary phase the glycogen degradation rate increases whilst the rate of glycogen accumulation increases. It can be calculated that if the rate of synthesis of glycogen is assumed to be the same in myxamoebae growing in the presence or absence of glucose and if the t_4 for glycogen degrada-

tion in myxamoebae grown in the presence of glucose is 36.5 h then the t_4 for glycogen degradation in myxamoebae grown in the absence of glucose would have to be 40 min in order to account for the lower cellular glycogen content. The observed t_4 for glycogen degradation in myxamoebae grown in the absence of glucose was 19.25 h. Thus we conclude that changes in the rate of glycogen degradation do not contribute significantly to the observed changes in glycogen content, and the assumption that changes in the glycogen content represent changes in the carbon flux through the glycogen synthetase reaction seems justified. It is clear from Table 3 that there is no correlation between the amount of glycogen synthetase possessed by a cell and the carbon flux into glycogen.

If the glycogen synthetase content of the myxamoebae does not change with alterations of carbon

Table 4. Amounts of glucose 6-phosphate and UDP-glucose in myxamoebae

Myxamoebae of strain Ax-2 were grown axenically to a cell density of 2.5×10^6 – 5.0×10^6 cells/ml, then were harvested rapidly, extracted, and assayed for glucose 6-phosphate and UDP-glucose, as described in the Materials and Methods section. Results are expressed as the mean $\pm$ S.E.M., and the number of determinations are given in parentheses.

Growth conditions	Glucose 6-phosphate content (nmol/ 10^8 cells)	UDP-glucose content (nmol/ 10^8 cells)
Axenic medium	2.35 ± 0.1 (3)	1.0 ± 0.11 (2)
Axenic medium + 86 mM-glucose	15.0 ± 1.4 (3)	3.26 ± 0.77 (3)

flux into glycogen the amount of enzyme present cannot control the rate of glycogen synthesis. Myxamoebae having a glycogen synthetase activity of 10 nmol/min per mg of protein should be capable of having a glycogen content of 9 mg of glucose equivalents of glycogen/ 10^8 cells if the intracellular environment of the enzyme was similar to that of our assay conditions. Clearly, this is not the case and glycogen synthetase cannot be operating at $V_{\max}$ intracellularly. Wright & Dahlberg (1967) described the isolation and characterization of glycogen synthetase from *D. discoideum* NC-4 cells that had been grown on bacteria. A number of differences exist between our results and those of Wright & Dahlberg (1967). Thus we report (Table 3) considerably higher specific activities and have been unable to stabilize our enzyme preparations completely. Also, whereas we can demonstrate excellent stoichiometric relationships between the amount of glucose transferred from UDP-glucose to glycogen and the amount of UDP formed (Table 2), Wright & Dahlberg (1967) only obtained good stoichiometry with stored preparations. Finally the enzymic activity detected by Wright & Dahlberg (1967) was glucose 6-phosphate-dependent whereas we find a much more complicated relationship between activity and glucose 6-phosphate amount (Fig. 3). Unfractionated extracts of axenically or bacterially grown myxamoebae of *D. discoideum* Ax-2 have considerable glycogen synthetase activity that is quite unaffected by the presence or absence of glucose 6-phosphate. However, addition of ATP to the reaction mixtures at physiological concentrations (Jones, 1970) causes a considerable inhibition that is particularly marked at physiological concentrations of UDP-glucose. Glucose 6-phosphate acts antagonistically to ATP and, at physiological concentrations of glucose 6-phosphate, the activity of glycogen synthetase would appear to depend on the glucose 6-phosphate/adenine nucleotide ratio (Fig. 3).

Both the UDP-glucose and glucose 6-phosphate contents of myxamoebae increased in media containing glucose (Table 4). This would be expected to

cause an increased rate of glycogen synthesis and hence an increase in the observed rate of glycogen accumulation (Table 3). The relationship between the UDP-glucose, glucose 6-phosphate and adenine nucleotide concentrations and the activity of glycogen synthetase is complex, and from our results it is impossible to correlate accurately the activities measured *in vitro* and those observed *in vivo*. Nevertheless it is clear that the rate of glycogen accumulation in myxamoebae grown in the presence of glucose of 1.7 nmol of glucose equivalents/min per mg of protein predicted from the glycogen synthetase activity determined *in vitro* at physiologically probable substrate, effector and inhibitor concentrations is in reasonably close agreement with the observed rate *in vivo* of 2.83 nmol of glucose equivalents/min per mg of protein. Such agreement supports our conclusion that the rate of glycogen synthesis in myxamoebae of *D. discoideum* is determined by the activity, and not the amount, of glycogen synthetase present.

It is of interest to determine whether glycogen synthesis is similarly controlled during the differentiation of myxamoebae and in the following paper (Hames, Weeks & Ashworth, 1972) we discuss this question.

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