Glycogen Synthetase and the Control of Glycogen Synthesis in the Cellular Slime Mould *Dictyostelium discoideum* during Cell Differentiation

By B. D. HAMES, G. WEEKS and J. M. ASHWORTH

Department of Biochemistry, School of Biological Sciences,
University of Leicester, Leicester LE1 7RH, U.K.

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1. The variation in cellular glycogen content of differentiating cells derived from myxamoebae that initially contained a wide range of glycogen contents (0.047–5.56 mg of glycogen/ 10^8 myxamoebae) has been studied. 2. Myxamoebae that initially contained 0.047–3.62 mg of glycogen/ 10^8 myxamoebae all gave rise to fruiting bodies that contained similar amounts of glycogen (0.06–0.11 mg of glycogen/ 10^8 cells) but myxamoebae that initially contained 5.56 mg of glycogen formed fruiting bodies containing 0.5 mg of glycogen/ 10^8 cells. 3. Despite the high net rate of glycogen disappearance (during cell differentiation) from cells that contained more than 2 mg of glycogen/ 10^8 cells initially, there were still significant variations in the rate of glycogen synthesis. The rate of glycogen synthesis reached a peak at the aggregation stage. 4. Evidence is presented showing that the rate of this synthesis of glycogen is controlled by factors other than the intracellular concentration of glycogen synthetase. 5. Our results are discussed in the context of the theory that the rates of glycogen synthesis and degradation act as a control mechanism for cell differentiation. 6. Criteria are discussed for deciding whether a biochemical event is causally or secondarily related to morphogenesis.

The glycogen content of the myxamoebae of the cellular slime mould *Dictyostelium discoideum* Ax-2 is a function of both the composition of the growth medium used (Ashworth & Watts, 1970) and the phase of the growth cycle (Weeks & Ashworth, 1972). Despite these changes in chemical composition, and associated changes in enzyme content (Quance & Ashworth, 1972) the myxamoebae differentiate normally to produce similar fruiting bodies by a similar time-schedule and in a similar morphogenetic sequence. Thus it is possible to examine the factors that control the cellular glycogen amounts during the differentiation of myxamoebae with either high or low glycogen contents. Wright *et al.* (1968) have suggested that the rate of both the synthesis and the degradation of glycogen alter, during cell differentiation of bacterially grown myxamoebae, in a way that can account for the observed amount of end-product saccharide (cellulose, trehalose and mucopolysaccharide) synthesis. Their model makes a number of predictions and these predictions can be tested by using myxamoebae of *D. discoideum* Ax-2 that contain different concentrations of glycogen.

Materials and Methods

Materials

Chemicals and radioisotopes were obtained from the sources described by Weeks & Ashworth (1972).

Glucose oxidase and hexokinase were obtained from Sigma (London) Chemical Co. Ltd., London W.5, U.K.

Methods

Growth and differentiation of myxamoebae. Myxamoebae of strains Ax-2 and NC-4 were grown, harvested and washed as described by Weeks & Ashworth (1972). The washed myxamoebae were deposited on well-washed Millipore filter supports at cell densities of 2.5×10^7 – 5×10^7 myxamoebae/filter (Sussman, 1966) and allowed to differentiate at 22°C. Fruiting bodies were fully formed 24 ± 2 h after deposition of the myxamoebae.

Determination of DNA. At various times during the differentiation sequence cells were harvested into ice-cold water. Usually sufficient cells were harvested to give a final suspension of 4×10^8 cells in 8 ml of water and these cell suspensions were then stored at –35°C. After being thawed, the cell suspensions were made up to 10 ml with water and 5 ml portions were sonicated with a 150 W M.S.E. ultrasonic disintegrator (peak-to-peak amplitude of $10 \mu\text{m}$) for either four 15 s periods (samples 0–16 h) or for 12 15 s periods (samples 16–30 h) with continuous cooling in an ice-salt bath to prevent excessive heating. The DNA content of sonicated cells prepared in this fashion was then determined by a modification of the procedure of Giles & Myers (1965): cold 50%

(w/v) trichloroacetic acid (1 ml) was added to 4 ml of extract, and the mixture was incubated for 1 h at 0°C. The resulting precipitate was centrifuged (1000g, 20 min). The supernatant was discarded and the pellet was washed with two 2 ml portions of cold 10% (w/v) trichloroacetic acid. The pellet was resuspended in 2.5 ml of 5% (w/v) trichloroacetic acid and heated at 90°C for 30 min to hydrolyse the DNA. The hydrolysate was centrifuged (1000g, 20 min) and to 1.6 ml of the clear supernatant was added 0.4 ml of 50% (w/v) HClO₄, 2.0 ml of 4% (w/v) diphenylamine in acetic acid and 0.2 ml of aqueous acetaldehyde (1.6 mg/ml). These reaction mixtures were then incubated at 30°C for at least 20 h and the difference in extinction at 595 and 700 nm was determined. The amount of DNA was estimated by comparison with solutions of known deoxyribose content that had been treated identically.

Determination of glycogen. Cell extracts were prepared as described above. A suspension of the extract (2 ml) and 1 ml of 90% (w/v) KOH were heated at 100°C for 20 min. Insoluble material was removed by centrifugation at 1700g for 10 min and extracted with 1 ml of 30% (w/v) KOH at 100°C for a further 20 min. After recentrifugation the two supernatants were combined and 0.2 ml of saturated Na₂SO₄ and 8 ml of ethanol were added. Incubation at 0°C for 10 min precipitated all the glycogen, which was collected by centrifugation at 38000g for 15 min. The pellet was dissolved in 3 ml of water and the glycogen was reprecipitated by addition of 6 ml of ethanol. Repetition of this precipitation procedure gave a white solid, which was finally dissolved in 2 ml of 0.5 M-H₂SO₄. This solution was heated at 100°C for 5 h to hydrolyse the glycogen and the pH was adjusted to 5–6 by repeated extractions with equal volumes of 10% (v/v) *NN*-diethylmethylamine in chloroform. The glucose concentration in this hydrolysate was then measured enzymically either by the glucose oxidase method of Dahlquist (1961) or by the hexokinase procedure of England & Randle (1967). Both methods gave identical results. Glycogen concentrations are expressed as glucose equivalents after hydrolysis/10⁸ cells.

Determination of the rates of glycogen degradation and synthesis. Myxamoebae were grown with continuous shaking in 65 ml portions of axenic medium containing 86 mM-[U-¹⁴C]glucose (390 µCi/86 mmol) in 250 ml Erlenmeyer flasks. The myxamoebae were harvested at a density of 2×10^6 – 4×10^6 myxamoebae/ml, then they were washed in cold water and allowed to differentiate at 22°C on Millipore filters at a density of 2.5×10^7 cells/filter. At various times the cells from six such filters were harvested into 60 ml of 1 mM-tris buffer, pH 7.4, containing 0.1 M-glucose, 0.6 g of NaCl/l, 0.75 g of KCl/l and 0.3 g of CaCl₂/l (Bonner, 1947; Marshall *et al.*, 1970). When necessary the developing cell aggregates were triturated before

dilution to a final volume of 60 ml so that the suspension consisted of clumps containing not more than five cells. The cell suspensions were then shaken in a water bath at 22°C for periods of up to 90 min. Disaggregated cells that had been incubated under these conditions did not lyse and appeared normal when viewed under the microscope. Samples (2 ml) of such suspensions were removed at 10 min intervals and added to 1 ml of 30% (w/v) KOH. Glycogen was isolated as described previously but with four cycles of alcohol precipitation before the final dissolution in 2 ml of water. Half of this solution was then added to 10 ml of a Triton X-100–toluene scintillation fluid (Fox, 1968) and counted for radioactivity at 62% efficiency in a Tracerlab scintillation counter. The remaining 1 ml portion was hydrolysed in 0.5 M-H₂SO₄ as described above and the neutralized hydrolysate was then assayed enzymically for glucose.

Determination of glycogen synthetase. The glycogen synthetase activity of cell-free extracts prepared from cells harvested (5 Millipores/3 ml) in 0.1 M-tris buffer, pH 7.5, containing 1.5 mM-EDTA and 2.5 mM-dithiothreitol was determined as described by Weeks & Ashworth (1972).

Results

Glycogen content of cells during differentiation

The change in glycogen content of myxamoebae grown in axenic media containing glucose during their differentiation is shown in Fig. 1 and similar results for myxamoebae grown in axenic media without added carbohydrate are shown in Fig. 2.

The percentage recovery of cells from Millipore filters varies during the differentiation sequence. For example, it is much easier to wash off the slug stages (14–16 h) by our harvesting procedures than it is to recover aggregating cells (0–7 h). To correct for this inherent variability the results have been normalized to glycogen contents/10⁸ cells by measuring, in the same sample, the amount of DNA and assuming (i) that the DNA content of 10⁸ myxamoebae is 17.9 µg and (ii) that the DNA content per cell does not change during differentiation. Both these assumptions are correct (B. D. Hames & J. M. Ashworth, unpublished work).

Myxamoebae that contained <0.1 mg of glycogen/10⁸ cells formed fruiting bodies containing 0.063 ± 0.003 mg of glycogen/10⁸ cells (Fig. 2). As the glycogen content of the myxamoebae was raised the rate of glycogen degradation during their subsequent differentiation increased so that fruiting bodies containing 0.083 ± 0.026 mg of glycogen/10⁸ cells are formed despite wide variations (1.49–3.62 mg of glycogen/10⁸ cells) in initial glycogen content (Fig. 1). Myxamoebae that initially contained 5.56 mg of glycogen/10⁸ cells (Fig. 1), however, formed fruiting bodies with

Fig. 1. Changes in the glycogen content of cells during differentiation

Myxamoebae were grown in axenic medium containing glucose and contained initially, per 10^8 myxamoebae: ■, 1.49 mg of glycogen; ●, 1.98 mg of glycogen; ○, 2.67 mg of glycogen; ▲, 3.62 mg of glycogen; △, 5.56 mg of glycogen.

significantly more (0.5 mg of glycogen/ 10^8 cells) than this, although the rate of glycogen degradation during differentiation of these cells was the highest we observed.

Determination of the rate of glycogen synthesis during differentiation

The decrease in glycogen content during the differentiation of myxamoebae that contained 1.49–5.56 mg of glycogen/ 10^8 myxamoebae (Fig. 1) may be the result of two rates, one of synthesis and the other of degradation (Wright & Dahlberg, 1967). We have therefore determined the absolute rate of degradation of [^{14}C]glycogen in differentiating cells. Cells that initially contained 2.63 mg of [^{14}C]glycogen/ 10^8 myxamoebae (see Fig. 1, points shown by ○) were allowed to differentiate for appropriate times. The cells were harvested and then incubated in the presence of [^{12}C]glucose as described in the Materials and Methods section before measurement of the radioactivity of the glycogen (Fig. 3).

Fig. 2. Changes in the glycogen content of cells during differentiation

Myxamoebae were grown in axenic medium and contained initially, per 10^8 myxamoebae: ○, 0.046 mg of glycogen; ●, 0.074 mg of glycogen.

The absolute rate of glycogen degradation was calculated by multiplying the glycogen content and the slopes of the lines of Fig. 3. The difference between the absolute rate of glycogen degradation, so determined, and the net rate of glycogen degradation shown in Fig. 1 (○) represents the rate of glycogen synthesis. The apparent rate of glycogen synthesis increased to a maximum at 12 h and then fell during the latter stages of differentiation (Fig. 4). To facilitate comparison of our results with those of Marshall *et al.* (1970), the time-period designated 'culmination' by these workers has been indicated on Fig. 4.

Glycogen synthetase activities of cells during differentiation

During the differentiation of axenically grown myxamoebae there was a continuous decrease in the activity of the glycogen synthetase, the activity at the end of differentiation being only half the activity at the onset (Fig. 5). These results were qualitatively and quantitatively different from the activities of glycogen synthetase of bacterially grown myxamoebae reported by Wright & Dahlberg (1967), who showed that the activity of the glycogen synthetase increased tenfold during aggregation, and then decreased tenfold during the remainder of the differentiation period. Therefore we also assayed the glycogen synthetase activity of bacterially grown strain Ax-2 during differentiation (Fig. 5). The change in activity of the enzyme during differentiation was qualitatively

Fig. 3. Determination of the rate of glycogen degradation during differentiation

Myxamoebae were grown in axenic medium + 86 mM- $[U-^{14}C]$ glucose and contained initially 2.6 mg of $[^{14}C]$ glycogen/ 10^8 cells. Cells were allowed to differentiate for $\circ$, 8 h; Δ , 12 h; $\blacktriangle$, 14 h; $\bullet$, 20 h. They were then harvested, and the aggregates were broken up. The cells were then resuspended in a glucose-salts medium at pH 7.4 and incubated at 22°C. Samples were taken at intervals and the glycogen was extracted for measurement of its radioactivity. We show representative results from three separate experiments; the 14 h and 20 h results come from the same experiment. Each determination represents the mean of four independent assays and the limits show the S.E.M.

similar to that reported earlier (Wright & Dahlberg, 1967), although we only observed a twofold increase in activity at aggregation. Possible reasons for these discrepancies have been discussed in relation to the activity of this enzyme during growth (Weeks & Ashworth, 1972).

Discussion

The kinetic model of Wright *et al.* (1968) is based on the idea that 'under the steady-state conditions of the living cell and over the time-periods normally involved in the process of differentiation changes in the levels of some essential enzymes are not significant with respect to controlling the metabolite flux necessary for the accumulation of specialized end products' (Wright, 1970). The model makes a number of assumptions. One of these is that at a critical point

Fig. 4. Variations in the rate of glycogen synthesis during differentiation of myxamoebae that initially contained 2.6 mg of glycogen/ 10^8 cells

Rates were calculated as the difference between the absolute rates of glycogen degradation (Fig. 3) and the apparent rate of glycogen degradation ($\circ$, Fig. 1). The bar denotes the 'culmination' period.

during the differentiation an increased availability of glucose 1-phosphate (caused by an enhanced rate of glycogen breakdown) causes an enhanced rate of UDP-glucose formation (without the necessity for any increase in UDP-glucose pyrophosphorylase content) and that this in turn produces a UDP-glucose pool sufficiently increased in size to account for the observed amount of end-product saccharide synthesis. Wright *et al.* (1968) explicitly assume that: $d[\text{glucose 1-phosphate}]/dt = k_2 \cdot [\text{soluble glycogen}] - V_{\text{UDPG}}$ where k_2 is a constant and V_{UDPG} is the rate of UDP-glucose synthesis. Their model predicts that to achieve this increased availability of glucose 1-phosphate there should be a threefold increase in the rate of both degradation and synthesis of glycogen at this critical time. Marshall *et al.* (1970) have reported that this change is found during the culmination stage of the differentiation of bacterially grown myxamoebae. Our experiments were designed to test some of the assumptions and predictions of this model.

Although there was a net decrease during differentiation in the glycogen of myxamoebae that initially contained 1.49–5.56 mg of glycogen/ 10^8 cells (Fig. 1) there was considerable glycogen synthesis, and the rate of this synthesis increased to a maximum

Fig. 5. Glycogen synthetase activity of strains NC-4 and Ax-2 during differentiation

Strain NC-4 and Ax-2 were allowed to differentiate at a cell density of 7×10^7 and 4×10^7 cells/filter respectively. At the indicated times, 2×10^8 cells were washed off the filters into 3 ml of 0.1 M-tris-chloride (pH 7.5)–0.0015 M-EDTA–0.0025 M-dithiothreitol. Suspensions were stored at -65°C until the next day and then the cells were disrupted by controlled sonication as described in the Materials and Methods section. The glycogen synthetase activity of the 5000g supernatant fraction was determined by measuring glycogen-dependent UDP formation for strain Ax-2 (●) and for strain NC-4 (○) and by $[\text{U-}^{14}\text{C}]$ glucose incorporation into glycogen (Δ), as described in the Materials and Methods section.

at 12h. However, the specific activity of glycogen synthetase was not increasing but decreasing during this time (Fig. 5). Further, there was at all times apparently an excess of enzyme capacity. Thus at 12h the rate of glycogen synthesis observed was still only one-tenth that possible if the enzyme were operating under optimum conditions. Hence, there was no evidence to suggest that during differentiation, as during growth (Weeks & Ashworth, 1972) the rate of glycogen synthesis was controlled by the cellular content of the enzyme glycogen synthetase. This is consistent with the contention that 'changes in the levels of certain essential enzymes are not significant with respect to controlling the metabolite flux, necessary for the accumulation of specialized end products' (Wright, 1970).

However, not all our results were consistent with the kinetic model. During differentiation there could be a considerable fluctuation in the glycogen content of the cells, and comparison of Figs. 1 and 2 shows that the same morphogenetic stage (e.g. 12h) could be formed from cells performing net glycogenolysis or glycogen synthesis or with a constant net glycogen content. The kinetic model of Wright *et al.* (1968)

demands that the glycogen content of the cells during the early stages of differentiation should remain constant and then decrease during culmination. Wright (1967) even suggested that the timing and spatial organization of developmental events might be determined by the utilization of a specific amount of reserve material, the rate of removal of this material being the controlling factor for the whole of morphogenesis. Clearly, such a view is difficult to reconcile with the results shown in Fig. 1. The considerable variation possible in the glycogen content of differentiating cells is much more consistent with the results of White & Sussman (1961), who found that the pattern of changes in the glycogen content of bacterially grown cells was qualitatively very similar to that shown in Fig. 2. However, whereas they suggested that the initial decrease was caused by the final utilization of ingested bacterial glycogen our results with axenically grown myxamoebae imply that this decrease is likely to be a genuine developmentally controlled event.

Another of the assumptions of the kinetic model is that there is no gluconeogenesis during differentiation, and Cleland & Coe (1969) showed that the

amount of gluconeogenesis during the differentiation of bacterially grown myxamoebae was slight. However, our results suggest that during differentiation there can be a significant increase in the glycogen content of the cells that initially have a low glycogen content (Fig. 2). This increase is caused by gluconeogenesis, because there is a concomitant increase in the total anthrone-positive material during the first 15 h (B. D. Hames & J. M. Ashworth, unpublished work).

Finally, the kinetic model requires that the rates of glycogen synthesis and degradation increase between aggregation and culmination and results that support this prediction have been reported (Marshall *et al.*, 1970). We have determined the rate of glycogen degradation in cells containing a relatively high initial glycogen content by a procedure identical with that of Marshall *et al.* (1970). This procedure involves resuspending developing cells in a glucose-salts medium and measuring the rate of the ensuing loss of radioactivity from the glycogen. We assume that the measured degradation rate is similar to that of the developing cells although, because the cells cease to differentiate when washed off the Millipore filters, this may not be strictly correct. Unfortunately, it is impossible to perform experiments of this kind with cells on the Millipore filters, since we cannot ensure that all of such cells are exposed to the same concentration of non-radioactive glucose. However, it is unlikely that any gross change occurs in the cells during the relatively short periods of incubation (90 min), as the cells recover and continue to differentiate if replaced on Millipore filters. Further, we are interested in changes in rates of degradation rather than in the absolute values, and it is probable that any metabolic changes introduced by this technique will be the same with different samples of cells. We find that cells with a relatively high initial glycogen content have a maximum rate of glycogen synthesis at aggregation (Fig. 4). This is in contrast with the results of Marshall *et al.* (1970) who found that in myxamoebae grown on bacteria the maximum rate was at culmination. Interestingly, the variation in the rate of glycogen synthesis during differentiation of cells with a high initial glycogen content (Fig. 4) parallels the variation in glycogen content of cells with a low initial content (Fig. 2). Thus it is possible that the net decrease in glycogen content of cells with high initial glycogen content masks the synthesis of a specific pool of glycogen that is of developmental significance.

The present paper and those preceding it (Edmundson & Ashworth, 1972; Ashworth & Quance, 1972; Quance & Ashworth, 1972; Weeks & Ashworth, 1972) are concerned with the nature of the biochemical events characteristic of cell differentiation in *D. discoideum*. In principle it might be expected that there would be two classes of such

events. One class would consist of those that are necessarily involved with the characteristic progressive changes in morphology that define the differentiation of the cells. The other class of biochemical events would then consist of those necessary to maintain the vital functions of the cells despite the changing environment and interactions imposed on the cells by the morphogenesis of which they are part. It is difficult to distinguish between these two classes of events. It is, however, important to make such a distinction, because only events of the first class can be said to 'cause' cell differentiation; events of the second class are merely secondary effects. However, because the initial physiological state of the differentiating myxamoebal cells can be altered by altering the conditions under which they are grown without thereby affecting the morphogenesis it is possible to distinguish between these two classes of event. If a presumptive developmental event alters during the differentiation of different myxamoebal populations then this event must be considered to be a class two event as defined above. If such an event does not alter then we here consider it to be a class one event. However, logically this criterion, although necessary, is not a sufficient proof that the event is of the first class, because there may be an infinite number of initial physiological states and, clearly, not all can be examined. We would like to restrict the term 'developmental programme' to those events that fall into class one, as defined above.

There is no reason to expect that the distinction we have made between classes of biochemical events corresponds necessarily to different levels of control (i.e. controls of the synthesis or of the activity of enzymes). Thus whereas the synthesis of, for example, *N*-acetylglucosaminidase cannot be said to be part of the 'developmental programme' its excretion may be (Quance & Ashworth, 1972). Similarly, the synthesis of alkaline phosphatase (Quance & Ashworth, 1972) by these same criteria may be regarded as part of the 'developmental programme'. The idea that the 'developmental programme' is controlled by the rates of glycogen synthesis and degradation, and hence the steady-state concentrations of glucose 1-phosphate and UDP-glucose, seems untenable. However, although the synthesis of UDP-glucose pyrophosphorylase (Edmundson & Ashworth, 1972; Ashworth & Watts, 1970) seems to be a class one event, it is clear that the initial enzyme content of the myxamoebae is itself sufficient to account for the rate of UDP-glucose production necessary for end-product saccharide synthesis. Part of the 'developmental programme' must therefore involve the requirement that a vast excess of this enzyme must always be present. Newell & Sussman (1970) have come to a similar conclusion on the basis of very different experimental evidence.

The biochemical problem posed by the cell

differentiation of *D. discoideum* thus becomes one of studying the interactions between different levels of control of what we have termed 'class one' events.

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