# Cell Wall Synthesis in *Dictyostelium discoideum*. II. Synthesis of Soluble Glycogen by a Cytoplasmic Enzyme*

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ABSTRACT: Glycogen synthesis from [$^{14}$C]uridine diphosphoglucose ([$^{14}$C]UDPG) is catalyzed by an enzyme in the 100,000$g$ pellet fraction of cells at all stages of differentiation. Stoichiometry of uridine 5′-diphosphate (UDP) released and [$^{14}$C]glucose incorporated into glycogen is achieved both with a once-washed pellet preparation and with a preparation purified 480-fold by adsorption onto and elution from amylose. Cellular glycogen remains at about 5% of the total dry weight until sorocarp construction; at this time the level drops to about 3% and remains at this value for at least 10 hr. Enzyme specific activity is high until the pseudoplasmodium stage of differentiation; during sorocarp construction it decreases, and then increases as the sorocarp matures. The enzyme is unstable at 30°, but can be stabilized by glycogen, glucose 6-phosphate (G-6-P), and UDPG. The $K_m$ of the enzyme for UDPG in the presence of G-6-P is about $4.2 \times 10^{-4}$ M; in the absence of G-6-P it is $4.7 \times 10^{-3}$ M.

Cell wall synthesis *in vitro* catalyzed by cell husk preparations of the cellular slime mold, *Dictyostelium discoideum*, has been described (Ward and Wright, 1965). The enzyme responsible for the synthesis of cell wall polysaccharides (a glycogen–cellulose complex) from [$^{14}$C]UDPG[1] at the terminal stages of differentiation is bound to the cell husk fraction; attempts to free it from primer have so far failed. It has recently been found that an enzyme located in the soluble fraction of the cell can also catalyze the incorporation of radioactive glucose from UDPG into insoluble cell wall material, if the latter is added as primer. Enzymatic and chromatographic analysis of the radioactive, insoluble product isolated showed it to be similar to that formed by the enzyme bound to cell wall preparations of young sorocarps; *i.e.*, most of the radioactive glucose is incorporated into $\alpha$-1,4 linkages of the cell wall material (Wright *et al.*, 1966). Further studies to be presented indicate that the cytoplasmic enzyme is identical with the one responsible for soluble glycogen synthesis (B. E. Wright and D. Dahlberg, unpublished data). The present communication deals with the characteristics of this enzyme, and with the composition of the 100,000$g$ pellet fraction during differentiation.

## Materials and Methods

[$^{14}$C]UDPG was purchased from New England Nuclear Corp. $\alpha$-Amylase (highest purity from hog pancreas), UDPG, and G-6-P were obtained from Sigma Chemical Co., and $\alpha$-glucosidase from Koch-Light Laboratories, Ltd. Oyster glycogen was obtained from Calbiochem, and potato amylose ("superlose") from Stein, Hall and Co., New York, N. Y. Protein was determined by the Lowry (1957) method.

Cellular glycogen was determined following rupture of the cells by physical or chemical means. Following treatment of the cells either by freezing and thawing or by passage through a French pressure cell, the 100,000$g$ pellet fraction was obtained (see Enzyme Preparation). After removal of protein by precipitation with TCA, the supernatant was heated with 1.2 volumes of 95% EtOH. Upon cooling, the glycogen precipitate was recovered by centrifugation, dissolved in water, and the precipitation procedure was repeated twice. Chemical disruption of the cells was accomplished by boiling in 30% KOH for 25 min. Following centrifugation to remove protein and cell debris, the supernatant was treated with 1.2 volumes of EtOH and the glycogen was precipitated three times. Oyster glycogen served as a control and was also alkali treated. Glycogen was measured by the phenolsulfuric acid method (Dubois *et al.*, 1956).

*Enzyme Preparation and Assay.* Cells of *D. discoideum* were harvested from 2% agar plates containing potassium phosphate buffer (pH 6.5, 0.01 M) and EDTA (0.001 M) (Ward and Wright, 1965). At the desired stage of development (usually early aggregation), a Tris-(0.1 M, pH 8.5) EDTA (0.025 M) buffer was used to harvest the cells; cell-free extracts were prepared either by freezing and thawing or by passage through a

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[1] Abbreviations used: UDPG, uridine diphosphoglucose; UDP, uridine 5′-diphosphate; G-6-P, glucose 6-phosphate; TCA, trichloroacetic acid.

French pressure cell (30,000 psi). Cell husk material was removed by centrifugation at 2000*g* for 10 min, and the supernatant fluid was centrifuged for 1 hr at 100,000*g*. The pellet was then washed once in the Tris-EDTA buffer and again sedimented at 100,000*g* for 1 hr. The enzyme is stable for at least 2 weeks if kept at −20°. Unless specified otherwise, enzyme activity was assayed in a total volume of 0.2–0.25 ml in the presence of Tris (0.025 M, pH 8.5), EDTA (0.005 M), [$^{14}$C]UDPG ($2 \times 10^6$ cpm/$\mu$mole, 0.003 M), G-6-P (0.005 M), 0.5 glycogen, and 50–100 $\mu$g of enzyme protein. The temperature and time of incubation vary and will be specified in each experiment. Incorporation of radioactivity into glycogen was linear over the time periods employed. The reaction was stopped by boiling for 3 min. UDP was determined spectrophotometrically by the pyruvate kinase assay (Leloir and Goldemberg, 1960). In order to determine radioactive glycogen, 10 $\mu$l of 5% carrier glycogen was added at the end of the incubation period. TCA (0.2 ml of 10%) was added and the supernatant was removed following centrifugation. The precipitate was washed once with 0.2 ml of 5% TCA and the wash was added to the first supernatant. Ethanol (2 ml of 95%) was added to the supernatant fluid (0.6 ml) which was brought to a boil and then cooled. The mixture was centrifuged and the supernatant fluid was discarded. The glycogen pellet was then dissolved in water and reprecipitated with ethanol. This procedure was repeated and the pellet was then transferred to a vial and counted in a Packard Tri-Carb liquid scintillation spectrometer.

TABLE I: Stoichiometry of UDP Release and [$^{14}$C]-Glucose Incorporation.

| Enzyme Preparation | $\mu$moles ($\times 10^{-4}$) of Glucose or UDP/$\mu$g of Protein | | | |
|---|---|---|---|---|
| | Day 1 | | Day 2 | |
| | Glucose | UDP | Glucose | UDP |
| Unwashed enzyme,[a] $3 \times 10^{-3}$ M UDPG | 3.5 | 11.6 | 4.0 | 8.8 |
| Unwashed enzyme, $1 \times 10^{-3}$ M UDPG | 3.4 | 10.8 | 3.4 | 8.4 |
| Washed enzyme,[a] $3 \times 10^{-3}$ M UDPG | 2.5 | 5.6 | 2.2 | 2.0 |
| Washed enzyme, $1 \times 10^{-3}$ M UDPG | 2.2 | 5.6 | 2.0 | 2.2 |
| Purified enzyme[b] | | | 98.0 | 136.0 |

[a] The assay was performed at 37° with a 60-min incubation; other assay conditions are described in Methods. Unwashed enzyme in the 100,000*g* pellet fraction was obtained from myxamoeba which had starved for 2 hr. Washed enzyme was sedimented a second time after homogenization in Tris-EDTA buffer. [b] The enzyme (aged amoeba) was purified as described in the text. UDP was assayed by the method of Leloir and Goldemberg (1960) and incorporation of [$^{14}$C]-glucose was determined as described in Methods. Incubation was 1 hr at 22°.

## Results

*Identification of Product and Stoichiometry.* In order to identify the product formed from UDPG, the following experiment was done. A typical reaction mixture was incubated 1 hr in the presence of [$^{14}$C]-UDPG (25,000 cpm). This substrate (18%) was incorporated into an ethanol-insoluble product (4500 cpm). Treatment of this material by $\alpha$-amylase solubilized 90% of the counts which, on chromatography (butanol–pyridine–water, 6:4:3), revealed two major peaks having the mobility of maltotriose and maltotetrose. The triose region had 65% of the counts (2600), 100% of which were converted to [$^{14}$C]glucose in the presence of $\alpha$-glucosidase.

An experiment was then done to determine the stoichiometry of UDP released and radioactive glucose incorporated into glycogen. Table I gives these data. Both washed and unwashed enzyme were used, and the assays were performed on 2 successive days. It can be seen that UDP release is higher on day 1 than day 2 with both enzyme preparations, and that washing the enzyme reduces reaction(s) resulting in UDP release not accompanied by glycogen synthesis. It has repeatedly been observed that UDP release decreases with the age of the enzyme preparation (to a variable degree, depending on the stage of differentiation). Washing and aging the enzyme, however, gives a preparation which may be stored for weeks at −20°, and with which good stoichiometry can be obtained (see day 2, washed enzyme).

*Purification of Glycogen Synthetase.* The enzyme was purified by adsorption on amylose and subsequent elution with glycogen (Algranati and Cabib, 1962). Aggregated cells were harvested in 0.1 M Tris and 0.025 M EDTA, frozen, thawed, and centrifuged at 2000*g* for 10 min. The supernatant solution was then centrifuged at 100,000*g* to obtain the pellet fraction. Aliquots of the enzyme (1.2 mg of protein) were mixed with various levels of amylose (in the presence of buffer) and incubated 5 min at 22°. The excess soluble enzyme was then removed by centrifugation and the amylose pellet was washed twice with cold buffer. Various levels of glycogen were then incubated with the amylose pellets for 5 min at 22° in order to elute the enzyme. These data are shown in Table II, from which it can be seen that the best purification was obtained using 10 mg of amylose/ml and 0.5 mg of glycogen/ml. Using these conditions, another enzyme preparation was purified with results shown in Table III. This enzyme was then examined for stoichiometry of UDP released and [$^{14}$C]glucose incorporation into glycogen ("purified enzyme," Table I). Such a purified enzyme showed a four- to fivefold dependency on G-6-P;

TABLE II: Optimal Conditions for Purification of Glycogen Synthetase.[a]

| Amt of Amylose Used for Trapping (mg/ml) | Glycogen for Elution (mg/ml) | Sp Act.[b] ($\times 10^{-5}$) | Purifcn |
|---|---|---|---|
| 5 | 2.0 | 5.6 | 15 |
| 10 | 2.0 | 34.0 | 94 |
| 20 | 2.0 | 34.0 | 94 |
| 10 | 0.5 | 46.0 | 130 |
| 10 | 2.0 | 34.0 | 94 |
| 10 | 5.0 | 17.0 | 46 |
| 0 | — | 0.36 | 0 |

[a] Unwashed pellet enzyme (0.1 ml, 1.2 mg of protein) was added to 0.1 ml of a solution containing 1–4 mg of amylose. Following a 5-min incubation at 22° to adsorb the enzyme, the amylose was washed twice with cold buffer. Enzyme was then eluted from the amylose by incubation for 5 min at 22° with 0.2 ml of a solution containing 0.05–0.5% glycogen. Purified enzyme was then assayed at the highest glycogen level. Cells at the aggregation stage of development were used. [b] [$^{14}$C]Glucose ($\mu$moles)/$\mu$g of protein per 1 hr.

the extent of this requirement varies with the enzyme preparation and temperature of assay.

*Enzyme Stability.* As has been found in a number of other systems (see Discussion), the slime mold enzyme-catalyzing glycogen synthesis is thermal labile. Preincubation experiments at various temperatures have shown that relatively little inactivation occurs over a period of 0.5–1 hr at 22° but that serious losses in activity occur at 30 or 37°. In the experiment summarized by Table IV, enzyme was preincubated for 1 hr in the absence or presence of G-6-P, UDPG, glycogen, or combinations thereof. Residual enzyme activity was then assayed by incubation at 22° for 0.5 hr. Clearly all three components protect the enzyme from inactivation at 30°. In order not to confuse the effects of a component as a stabilizer with its effect as a substrate or effector, experiments should be done at lower temperatures, thus minimizing inactivation.

*Glycogen, G-6-P, and UDPG Dependency.* The dependency of glycogen synthesis upon glycogen and G-6-P was examined at 22 and 37°. Figure 1A shows that, in the absence of G-6-P at 22°, the cellular level (double arrow) of glycogen as primer does not limit the rate of its synthesis. At 37°, however, it is interesting to note that saturation was not achieved. An obvious explanation is that, as glycogen levels increase, the amount of functional enzyme also increases due to its protection from heat inactivation. A comparable effect is noted in Figure 1B, in that less G-6-P is required for a given amount of glycogen synthesis at 22° than at 37°. It can also be seen that, at 37°, glycogen has a "sparing effect" on the requirement for G-6-P; that is, less G-6-P is required for a comparable activity at high glycogen levels. This is understandable, since both G-6-P and glycogen stabilize (Table IV). At either temperature, cellular levels of G-6-P would limit glycogen synthesis, and would affect the $K_m$ for UDPG (see below). The intracellular levels of glycogen and G-6-P have been determined (see Table VII, and Wright, 1966a).

The dependency of glycogen synthesis on UDPG concentration was examined in the presence and absence of G-6-P, which is known to lower the $K_m$ for UDPG in other systems (Kornfeld and Brown, 1962; Traut and Lipmann, 1963). The average $K_m$ values for a number of experiments are presented in Table V. In the presence of G-6-P and glycogen, the enzyme is stable (Table IV); $K_m$ values determined under these conditions are not affected by the temperature of incubation.

The data are not affected by the stage at which the enzyme was prepared. Cellular levels of UDPG have been determined, and clearly limit glycogen synthesis (see Wright, 1966b).

*Specific Enzyme Activity as a Function of Development.* In three experiments, glycogen synthesis was examined in the 100,000$g$ pellet fraction. Specific enzyme activities are summarized in Table VI. Peak

TABLE III: Purification of Glycogen Synthetase.

| Enzyme Source | Total Vol. (ml) | $\mu$moles Inc/ml | Total $\mu$moles Inc | % Recov[a] | Sp Act.[b] ($\times 10^{-5}$) | Purifcn |
|---|---|---|---|---|---|---|
| 2000$g$ supernatant | 36 | 0.587 | 21.1 | 100 | 2.7 | 1 |
| 100,000$g$ pellet | 12 | 0.630 | 7.56 | 36 | 10.9 | 4.04 |
| Eluted trapped enzyme[c] | 12 | 0.210 | 2.52 | 12 | 1300 | 481 |

[a] Recovery of activity in crude supernate taken as 100%. [b] The units are micromoles of [$^{14}$C]glucose per microgram of protein per hour. [c] Pellet enzyme (100,000$g$) after trapping and eluting from amylose.

TABLE IV: Stabilization of Enzyme from Thermal Inactivation.[a]

| Components Present during Preincubn | μmoles (× $10^{-3}$) of Glucose Inc in Final Incubn | % Original Act. |
|---|---|---|
| Not preincubated | 6.3 | 100 |
| G-6-P, glycogen | 6.1 | 97 |
| UDPG, glycogen | 6.0 | 95 |
| UDPG, G-6-P | 5.5 | 87 |
| Glycogen | 4.8 | 76 |
| UDPG | 3.8 | 60 |
| G-6-P | 2.5 | 40 |
| None | 0.9 | 14 |

[a] Preincubation was carried out for 1 hr at 30°. Missing components and radioactive UDPG were then added, making all tubes identical for the final incubation of 0.5 hr at 22°. The final concentration of each component was as follows: UDPG ($1 \times 10^{-3}$ M), G-6-P ($5 \times 10^{-3}$ M), and glycogen (5 mg/ml).

TABLE VI: Specific Activity of Enzyme during Development.[a]

| Stage | μmoles (× $10^{-3}$) of [$^{14}$C]Glucose/μg of Protein | | |
|---|---|---|---|
| | Expt I | Expt II | Expt III |
| Amoeba | 0.024 | | |
| Aggregation | 0.047 | | 0.020 |
| Late aggregation | 0.092 | | |
| Young pseudoplasmodium | 0.068 | | |
| Pseudoplasmodium | 0.022 | | 0.13 |
| Early preculmination | 0.012 | | |
| Preculmination | 0.006 | 0.012 | |
| Culmination | 0.008 | 0.015 | |
| Culmination sorocarp | | 0.007 | 0.015 |
| 2-hr sorocarp | | 0.008 | |
| 5-hr sorocarp | | 0.012 | 0.024 |
| 6-hr sorocarp | | 0.012 | |
| 8-hr sorocarp | | | 0.048 |

[a] The enzyme preparation and assay conditions are described in Methods. Incubation was at 22° for 1 hr.

activity is seen in the late aggregation stage of development, and minimal values are obtained for the 2-hr sorocarps. Specific enzyme activity again rises during maturation of the sorocarp.

*Pellet Composition During Development.* In connection with studies of enzyme specific activity during development, it was of interest to know the general behavior of the pellet fraction as a function of successive stages of morphogenesis. Table VII summarizes such a study and reveals that very little change occurs with respect to dry weight and polysaccharide content until sorocarp construction, at which time a drop is noted.

TABLE V: $K_m$ for UDPG in the Presence and Absence of G-6-P.[a]

| No. of Expt | G-6-P Level (M) | $K_m$ (M) | Std Dev |
|---|---|---|---|
| 4 | $5 \times 10^{-3}$ | $4.2 \times 10^{-4}$ | $\pm 2.8 \times 10^{-4}$ |
| 2 | $1 \times 10^{-4}$ | $2.0 \times 10^{-3}$ | $\pm 0.5 \times 10^{-3}$ |
| 3 | None | $4.7 \times 10^{-3}$ | $\pm 1.7 \times 10^{-3}$ |

[a] Assay conditions are the same as described in Methods except for varying UDPG concentrations. Incubation was for 1 hr at 22° in the absence of G-6-P, and at 22 or 37° in the presence of G-6-P. Pellet enzymes from amoeba or young sorocarp was used.

Toward the end of development a French pressure cell must be used to rupture the cells. This method was, therefore, compared to freezing in the same stage study (expt III). More pellet material was obtained, but the fraction of the pellet represented as polysaccharide decreased. This was explained in part by the fact that relatively more protein was released by the French pressure cell treatment. In order to verify the decrease in pellet material in young sorocarps ruptured by passage through a French pressure cell (expt II), a chemical method was used on whole cells (expt IV). These data confirmed the concentration of glycogen in the cell, as well as the decrease of this material in young sorocarps.

## Discussion

In view of the fact that the extreme thermal lability of the enzyme responsible for glycogen synthesis has been known for some time in mammalian systems (Leloir and Goldemberg, 1960), it is surprising that experiments have been routinely carried out at temperatures known to inactivate the enzyme. At 30°, striking and additive effects of G-6-P, UDPG, and glycogen on the stabilization of the enzyme are observed (Table IV, Figure 1). At 20°, when the enzyme is more stable, it is possible to minimize confusion arising from stabilization effects in the interpretation of experiments designed to obtain other kinds of information ($K_m$ values, the extent of activation by G-6-P, etc.).

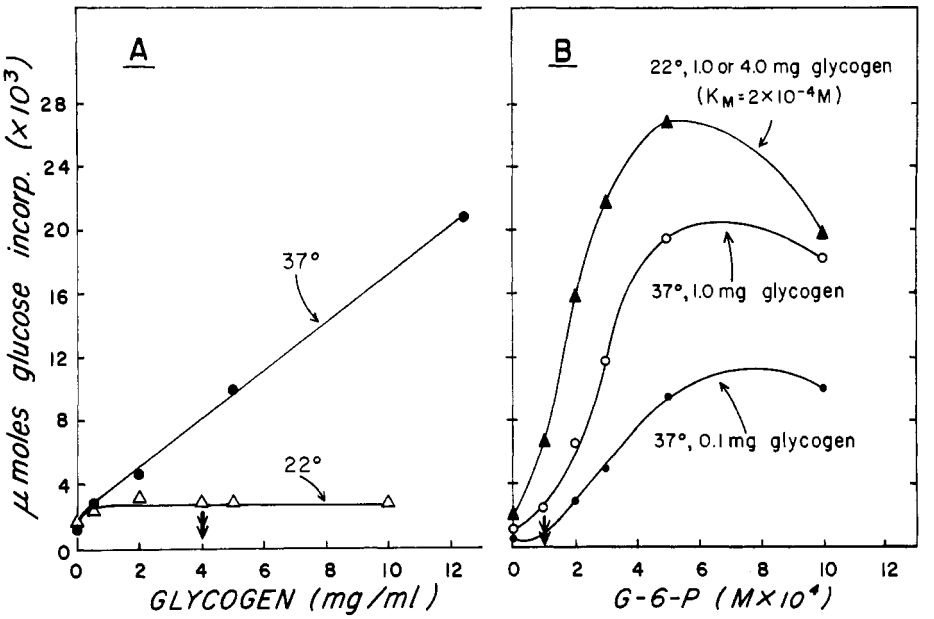


FIGURE 1: Glycogen synthesis as a function of glycogen concentration at 37 and 22°. (A) No G-6-P is present, and UDPG was present at $10^{-3}$ M; see Methods for other experimental details. Different enzyme preparations were used at the two temperatures. The cellular concentration of glycogen is indicated by a double-headed arrow. (B) UDPG was present at $10^{-3}$ M, and the glycogen level varied in each experiment; for other details, see Methods. The approximate concentration of G-6-P found in differentiating cells is indicated by a double-headed arrow.

TABLE VII: Composition of the 100,000*g* Pellet during Development.[a]

| Stage Study No. | Stage | Pellet as % Total Dry Wt[b] | Sugar as % Pellet Dry Wt[b] | Protein as % Pellet Dry Wt[b] | Sugar as % Total Dry Wt: Physical Methods[b] | Sugar as % Total Dry Wt: Chemical Methods[c] |
|---|---|---|---|---|---|---|
| I | Amoebae | 18 | 27 | | 4.7 | |
| II | Amoebae | 17 | 33 | | 5.8 | |
| III | Amoebae | 17 (30) | 33 (24) | 40 (63) | 5.5 (7.3) | |
| I | Late aggregation | 18 | 28 | | 4.9 | |
| I | Preculmination | 17 | 24 | | 4.0 | |
| III | Preculmination | 13 (27) | 24 (12) | 59 (65) | 3.2 (3.3) | |
| II | Late preculmination | 17 | 26 | | 4.4 | |
| I | Culmination | 15 | 27 | | 4.0 | |
| IV | Late culmination | | | | | 5.1 |
| II | Late culmination | (28) | (16) | | (4.3) | |
| IV | Culmination sorocarp | | | | | 5.5 |
| IV | 2-hr sorocarp | | | | | 5.0 |
| II | 2-hr sorocarp | (21) | (16) | | (3.4) | |
| IV | 5-hr sorocarp | | | | | 3.1 |
| IV | 9-hr sorocarp | | | | | 2.8 |

[a] At the indicated stages of development in three stage studies the 100,000*g* pellet fraction was prepared and analyzed for dry weight of polysaccharide and protein contents (see Methods). [b] Cells were ruptured by freezing for obtaining all values except those in parentheses, which were obtained from cells ruptured by passage through a French pressure cell. [c] Cells were heated 25 min in 30% KOH in steam, the cell debris was centrifuged and to the supernatant was added 1.2 volumes of 95% ETOH. The ETOH mixture was brought to boiling, then frozen overnight (−20°). The glycogen was further purified by twice precipitating it from water.

About 80% of the pellet glycogen fraction can be accounted for as polysaccharide and protein; the remainder is at least in part RNA, which has also been shown to be present. The per cent of the pellet glycogen that was alkali soluble by the procedure of White and Sussman (1963) was about the same at all stages of development. Since their fraction (isolated from whole cells) peaked in concentration at the pseudoplasmodium stage, it is apparently unrelated to ours (Table VII).

The size of the glycogen pellet decreases during the fruiting process, and may serve as the final energy source during morphogenesis. The fact that the cellular concentration of $P_i$ rises to very high levels at the later stages of development (0.05 M; Gezelius and Wright, 1965) is consistent with this observation, in that phosphorylase activity would be stimulated (Brown and Cori, 1961). The minimal enzyme specific activity also occurs in the early stages of sorocarp construction (Table VI). Glycogen degradation could result in the release of enzyme from this fraction, and aid its transfer to the cell wall, possibly accompanied by glycogen as a primer (Wright *et al.*, 1966). Assuming no significant compartmentalization in the intact cell, the data presented in this paper indicate that observed changes in the levels of UDPG and G-6-P during differentiation will influence the rate of soluble glycogen synthesis.

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