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Glycogen Turnover in *Dictyostelium discoideum**

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ABSTRACT: The rates of soluble glycogen synthesis and turnover have been determined *in vivo* at two stages of differentiation in *Dictyostelium discoideum*. Calculation of the rate of soluble glycogen synthesis was based upon the specific radioactivity of intracellular uridine diphosphate [^{14}C]glucose ([^{14}C]UDPG) following exposure of the cells to [^{14}C]glucose, and upon the increase with time in radioactivity of the glycogen pool. Glycogen turnover was determined by the exposure of cells which were previously labeled with [^{14}C]-glucose to [^{12}C]glucose, in order to determine the change in glycogen specific radioactivity with time. The rate of glycogen synthesis was found to be 0.034 and 0.160 μmole per min per ml of packed cells at aggregation and culmination, respectively; for glycogen turnover, the comparable values were 0.046 and 0.130 μmole per min per ml of packed cells. These rates are in excellent agreement with each other and with the rate of UDPG synthesis determined previously, thus substantiating two predictions of a kinetic model simulating glycogen metabolism during differentiation in this system.

The organism *Dictyostelium discoideum* grows as individual amoebae by engulfing bacteria. When the food supply is exhausted, the cells aggregate into a multicellular unit which maintains itself in the absence of exogenous nutrients for the remainder of the life cycle. Differentiation is completed within 24 hr at 23°, and is characterized by an orderly sequence of morphological changes which ultimately results in the formation of a mature fruiting body, or sorocarp, during a process called culmination.

It has been shown that, prior to sorocarp construction, the soluble glycogen pool (100,000*g* pellet fraction) accounts for about 5% of the total dry weight from the beginning of the differentiation cycle (amoeba stage) to culmination (Wright and Dahlberg, 1967), after which insoluble glycogen accumulates complexed to cellulose (Wright, 1966). Although the relative rates of soluble glycogen synthesis and degradation at aggregation and culmination had not been studied directly in the laboratory, the rates *in vivo* were predicted by a kinetic model which simulates glycogen metabolism during differentiation (Wright *et al.*, 1968; Wright, 1968). Although the specific activity of glycogen synthetase, the enzyme which catalyzes glycogen synthesis from UDPG,[1] decreases about tenfold *in vitro* between the aggregation stage and culmination (Wright and Dahlberg, 1967), this information was not incorporated in the model. In fact, the model predicted a threefold increase in the rates of glycogen synthesis and degradation *in vivo* over this same time period.

The purpose of this investigation was to determine experimentally the rates *in vivo* of glycogen synthesis and degradation at aggregation and at culmination. The information gained has enabled us to determine the relevance *in vivo* of flux values predicted by the kinetic model.

Materials and Methods

Materials. Maltase (type III), β-amylase (type II-B), bovine albumin (fraction V powder), and MES were all purchased from the Sigma Chemical Co. [U-^{14}C]Glucose (1.8×10^7 dpm/μmole) was purchased from New England Nuclear and oyster glycogen from Calbiochem. Sterile disposable petri dishes (150 × 50 mm) were obtained from Fisher Scientific.

Preparation of Cells. D. discoideum strain NC-4 was grown as previously described by Liddel and Wright (1961). The washed cells, essentially free of bacteria, were spread onto 2%

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[1] Abbreviations used are: UDPG, uridine diphosphate glucose; MES, 2-(*N*-morpholino)ethanesulfonic acid; PEI-cellulose, polyethyleneamine cellulose.

agar sheets containing either 0.01 M phosphate buffer (pH 6.5) and 0.001 M EDTA or 0.01 M MES buffer (pH 6.5) and allowed to differentiate at 15° to the desired stage of development. At this time, the cells were harvested with Bonner's salt solution (Bonner, 1947) in either 0.001 M Tris-HCl (pH 7.4) (Bonner's-Tris), or in 0.001 M MES–NaOH (pH 6.5) (Bonner's-MES). Homogeneous suspensions were routinely prepared by repeatedly pipetting the cells through a wide-bore 10-ml pipet.

Dry Weight and Cell Volume Determinations. Cells were prepared as described above and allowed to differentiate at 23°. At each stage used, *i.e.*, aggregation, culmination, and sorocarp, the cells were harvested in cold distilled water and suspended in a final volume of 11 ml. Aliquots (2 ml) were taken in duplicate for each packed cell volume determination and in triplicate for each dry weight determination. That no significant cell lysis occurred under these conditions was shown in a separate experiment.

The pellet volume was determined for cells which had been centrifuged in graduated test tubes (Fisher No. 5-663) at 1000*g* for 15 min; this volume was then corrected to represent only that percentage of the pellet volume actually consisting of cells (Barravecchio *et al.*, 1969). Samples for dry weight determinations were dried at 100° until three consecutive weighings were the same (about 36 hr).

Identification of Soluble [^{14}C]Glycogen. The cells were prepared and labeled (see turnover experiments) at the aggregation stage. The washed cell suspension (2 ml) was spread onto each of two phosphate–EDTA agar plates and the cells were allowed to differentiate at 15°. One plate was harvested at the aggregation stage and one at culmination, each with 1.0 ml of distilled water. The cells were transferred to a centrifuge tube and frozen.

Glycogen was isolated (see below) from the thawed cells at each stage, purified, and then dissolved in 0.2 ml of 0.05 M acetate buffer (pH 4.7); 0.1 ml was transferred to a vial for counting and the remainder was incubated with 0.1 ml of β-amylase (1 mg/ml) for 4 hr at 23°. The reaction was stopped by heating each tube at 100° for 5 min.

To ensure precipitation of any residual radioactive glycogen, 30 μl of 5% glycogen and 1.2 volumes of 95% ethanol were added. The tubes were heated to 100° and centrifuged at 0° for 10 min at 2000*g*. The supernatant fractions (containing [^{14}C]maltose) were saved for chromatography and the precipitated glycogen samples dissolved in 0.1 ml of the acetate buffer and counted in a Beckman LS-200 scintillation counter (efficiency 85% with ^{14}C).

In order to determine the per cent conversion of glycogen into maltose by β-amylase, the supernatant portions along with known glucose and maltose were chromatographed on Whatman No. 1 paper in a butanol–acetate–95% ethanol–water (5:4:3:2, v/v) solvent system for 24 hr by the ascending technique. Location of the sugars was detected by treating the paper with alkaline $AgNO_3$ reagent (Trevelyan *et al.*, 1950). Areas corresponding to the location of authentic maltose were eluted for 24 hr in distilled water. The eluates were lyophilized and dissolved in 0.8 ml of distilled water, and 0.2 ml of each sample was counted. The remainder of each sample was incubated with 0.2 ml of maltase (100 mg/ml) in 0.2 M phosphate buffer (pH 6.9) for 3 hr at 37°.

The reaction mixture (now containing [^{14}C]glucose) was applied to Whatman No. 1 paper with the appropriate controls, and chromatographed as before. The control strips were developed and the areas with the same mobility as the glucose control were eluted for 24 hr with distilled water. The eluates were lyophilized, dissolved in 0.2 ml of distilled water, and counted.

Glycogen Synthesis. In order to determine the rate of glycogen synthesis, it was necessary to isolate both glycogen and its precursor, UDPG.

LABELING CONDITIONS. Cells which had been allowed to differentiate to the aggregation stage or to culmination were harvested in either Bonner's-Tris or Bonner's-MES such that 1 ml of the resulting cell suspension was equivalent to 0.1 ml of packed cells. Aliquots (2–5 ml) of this suspension were transferred to 50-ml erlenmeyer flasks and incubated at 23° on a shaker in the presence of 0.02 mM [U-^{14}C]glucose (1.8×10^7 dpm/μmole). Flasks were removed, two at a time, at intervals 10 min apart; the maximum incubation time was 50 min. The cells from one flask at each time interval were used for glycogen isolation and the cells from the other flask for UDPG isolation.

GLYCOGEN ISOLATION. The cells were killed by the addition of an equal volume of 60% KOH and extraction was allowed to proceed for 30–60 min at 4°. The mixture was centrifuged, the supernatant portion was retained, and the pellet was reextracted once with 30% KOH. Oyster glycogen (1.0 mg) was added to the combined supernatant portions and 1.2 volumes of 95% ethanol were added; the solution was then boiled, cooled, and stored overnight at −20°. The glycogen was purified as described previously (Wright and Dahlberg, 1967).

[^{14}C]UDPG ISOLATION. Cells were killed by the addition of an equal volume of 0.7 M $HClO_4$ and extraction was carried out for 30–60 min at 4° with occasional stirring. The suspension was centrifuged at 12,000*g* for 5 min, the supernatant was saved, and the pellet was reextracted once with 1.0 ml of 0.35 M $HClO_4$. The combined supernatant portions were neutralized with 5 N KOH. UDPG was isolated by adsorption and elution from activated charcoal (Pannbacker, 1967) and separated by thin-layer chromatography on PEI-cellulose sheets (Randerath and Randerath, 1965). The UDPG area was cut out and eluted for 1 hr with 1.0 ml of 0.7 M $MgCl_2$ in 0.02 M Tris (pH 7.4) at 23°. The specific radioactivity of UDPG was determined by counting 0.5 ml of the eluate in 10 ml of a dioxane gel (Gordon and Wolfe, 1960), and relating this value to the A_{260} of the eluate.

CALCULATION OF THE RATE OF GLYCOGEN SYNTHESIS. The micromoles of glucose incorporated into glycogen were calculated from the specific radioactivity of [^{14}C]UDPG. The rate of glycogen synthesis could then be determined from the increase with time in the μmoles of glucose incorporated and was expressed as μmoles of glucose incorporated per minute per milliliter of packed cells.

Glycogen turnover was studied by first labeling the glycogen pool, and subsequently following the rate at which the glycogen specific radioactivity decreased in the presence of unlabeled glucose.

PRELABELING. Cells which had been allowed to develop to the desired stage of differentiation were harvested and suspended in buffer as specified; 40 ml of this suspension ($\simeq$4 ml of packed cells) was transferred to a 250-ml erlenmeyer flask and [U-^{14}C]glucose was added to give a final concentration of 0.01 μM (sp act. 1.8×10^7 dpm/μmole), or as

specified in each experiment. The suspension was shaken for 45 min or 60 min at 23° on a Fisher rotator shaker. The labeled cells were washed three times in the same buffer used for their harvest by repeated centrifugation at 1500*g* for 60 sec at 3°. The cells were resuspended in buffer such that a packed cell volume of 0.1–0.15/ml was obtained, and were spread onto sterile disposable petri dishes (2 ml/plate) containing 22.5 ml of 1% agar in either Bonner's-Tris (pH 7.4) or Bonner's-MES (pH 6.5) as specified. These plates were routinely dried for 48 hr at 23° prior to use. The cultures were incubated at 15° until the desired stage was reached.

[^{12}C]Glucose chase. In order to determine the rate of glycogen turnover, chase experiments using [^{12}C]glucose were carried out using labeled cells which had been allowed to differentiate to either the early aggregation stage or to preculmination on nonnutrient agar buffered with either Bonner's-MES or Bonner's-Tris. At the desired stage of development, the plates were flooded with 2.5 ml of a glucose solution to give a final concentration of 10^{-2} or 10^{-3} M, as specified. Under these conditions, the glucose solution was absorbed by the agar. Alternatively, cells were harvested into 50-ml erlenmeyer flasks in Bonner's-Tris or in Bonner's-MES when the desired stage was reached, and glucose was added to the liquid cell suspension to give comparable glucose concentrations. The cultures were incubated at 23° for varying chase times (the liquid cultures on a shaker), harvested, and transferred to conical centrifuge tubes for the isolation of glycogen and protein.

For studies at aggregation, the cells were chased over a period of time during which they differentiated only to late aggregation or the early pseudoplasmodium stage; in the experiments beginning with preculminating cells, differentiation was allowed to proceed to culmination only.

Isolation of glycogen and protein. The cells, either frozen or live, were disrupted by grinding with 10% trichloroacetic acid (v/v) in an ice bath or by the addition of an equal volume of 60% KOH and extraction at 100° for 30 min. The suspensions were centrifuged at 2000*g* for 10 min at 3° in an International refrigerated centrifuge, Model PR-2. The supernatant portions were transferred to conical tubes and 1.2 volumes of 95% ethanol were added. After the solutions were brought to boiling they were allowed to stand for 24 hr at −20° to aid in the recovery of glycogen. The subsequent purification procedure was as described by BeMiller (1965), except that only three ethanol precipitations were routinely used prior to dissolving the glycogen in distilled water. Under these conditions lithium bromide was not required to induce flocculation.

For determination of specific radioactivity, half of each sample was counted and the remainder was used for the colorimetric determination of reducing sugar according to the phenol–sulfuric acid method of Dubois *et al.* (1956).

NaOH (1 ml of 1 N) was added to the pellet portion which contained both trichloroacetic acid insoluble protein and cell debris. After mixing thoroughly, the samples were centrifuged at 3° for 20 min at 2000*g*. The supernatant portion was transferred to conical centrifuge tubes, and the pellet, after being reextracted once with NaOH, was discarded. The protein was purified partially by repeating the precipitation from NaOH solutions with trichloroacetic acid three times. The purified trichloroacetic acid insoluble protein was dissolved in 0.2 ml of 1 N NaOH and 0.1 ml was transferred to vials for counting. The remainder was used for determination of protein concentration according to the method of Lowry *et al.* (1951), using bovine albumin as the reference standard.

Calculation of glycogen turnover rate. Data obtained from each chase experiment were subjected to statistical treatment using conventional methods for linear, quadratic, and cubic regression analysis (Steel and Torrie, 1960). The regression coefficients obtained were tested for significance; the linear fit was best in each case. The curves were constructed according to the slope and Y intercept values generated in the analysis and were extrapolated to show the time at which the glycogen specific radioactivity was half of its initial value. One-half of the concentration of intracellular glycogen at the stage of differentiation being investigated was then divided by this time in minutes to give an estimate of the turnover rate in micromoles of glucose per minute per milliliter of packed cells.

Results

Dry Weight and Cell Volume Determination. In order to calculate flux values and relate these to the characteristics of relevant enzymes and to the concentration of metabolites *in vivo*, all values have previously been expressed in terms of glucose equivalents per milliliter of packed cells at aggregation (Wright *et al.*, 1968). For these calculations, the ratio of dry weight to packed cell volume was assumed to be 100 mg/ml at aggregation. The percentage of the cell pellet volume actually consisting of cells has recently been determined (Barravecchio *et al.*, 1969), and using these values, the average ratio of dry weight to packed cell volume at aggregation (six determinations) was found to be 172 ± 27 mg per ml. These data were used in the calculation of the rate of glycogen synthesis and for the determination of the concentration of glycogen at aggregation and culmination. The glycogen concentrations were required for the calculation of turnover rates. It was previously reported (Wright and Dahlberg, 1967) that glycogen represents approximately 5% (≃28 μmoles) of the dry weight at both aggregation and at culmination, the two stages of differentiation studied in this report. At aggregation, the corrected value is therefore 28/(100/172), or 48.5 μmoles/ml of packed cells, and at culmination, it is 28/(100/172) × 86% (to account for the decreased dry weight at culmination) or 41.5 μmoles/ml of packed cells.

Identification of Radioactive Polysaccharide Fraction. Following the exposure of intact cells to [U-^{14}C]glucose, the radioactive polysaccharide fraction was isolated at two stages of differentiation (see Methods) and further identified by enzymatic and chromatographic analysis. As can be seen from Table I, β-amylase treatment of this fraction resulted in 40–50% conversion into maltose. The [^{14}C]maltose was identified chromatographically and by conversion to [^{14}C]glucose in the presence of maltase. In a separate experiment it was found that the per cent of counts released by β-amylase digestion was twice as high as the per cent of sugar released, indicating periferal labeling of the glycogen molecule. The results of the enzymatic digestion of the radioactive polysaccharide fraction synthesized *in vivo* parallel that expected from the digestion of known glycogen (Stetten and Stetten, 1960). Comparable results were obtained in the analysis of radioactive glycogen synthesized *in vitro* (Wright and Dahlberg, 1967).

Glycogen and Protein Specific Radioactivity from Aggre-

TABLE I: Identification of ^{14}C-Labeled Polysaccharide Material by Enzymatic Digestion and Chromatography. Conditions as Described under Methods.

Stage	Enzyme	Substrate	Product	cpm[a]	% Conversion
Aggregation		[^{14}C]Polysaccharide		3,092	
	β-Amylase	[^{14}C]Polysaccharide	Maltose	1,484	48
	Maltose	Maltose	Glucose	1,187	80
Culmination		[^{14}C]Polysaccharide		31,370	
	β-Amylase	[^{14}C]Polysaccharide	Maltose	12,548	40
	Maltose	Maltose	Glucose	10,917	87

[a] Total counts (corrected for background).

gation to Culmination. Cells at the early aggregation stage of development were exposed to [U-^{14}C]glucose, washed, and replated (zero time). Samples were harvested at the indicated time periods during differentiation until culmination (see Figure 1). The specific radioactivity of glycogen was found to be essentially constant during this portion of the differentiation process, with either low or high initial glycogen specific radioactivity, and using either the KOH or trichloroacetic acid method for the initial extraction.

Protein specific radioactivity was lower than that of glycogen under both labeling conditions and increased slightly as differentiation proceeded. A significant contribution to glycogen from protein *via* gluconeogenesis would, therefore, be detected under these experimental conditions. Apparently a greater fraction of the glycogen pool was labeled in the presence of the higher glucose level.

Glycogen Synthesis. Cells were exposed to [^{14}C]glucose over a period of time during which the specific radioactivity of intracellular UDPG was relatively constant and the radioactivity of the glycogen pool was increasing in a linear fashion (see Figure 2). Under these circumstances it was possible to calculate the rate of glycogen synthesis; similar rates were obtained under conditions where pH and buffer varied. The data are summarized in Table II, and show a significant increase in the rate of glycogen synthesis from the aggregation to the culmination stage of differentiation.

Incubation periods of up to 50 min in liquid were not detrimental to the cells; differentiation progressed normally upon the subsequent transfer of such cells to agar plates.

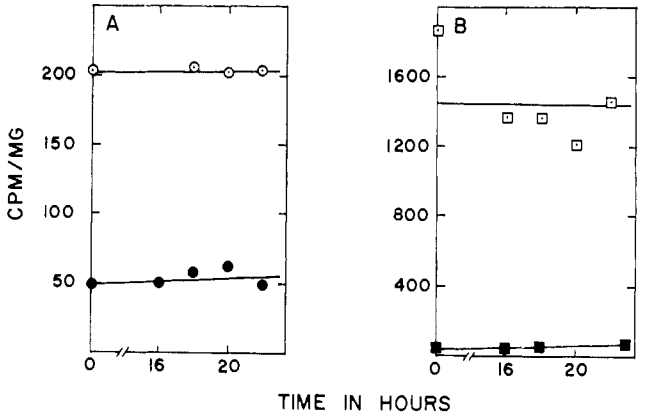

FIGURE 1: Glycogen and protein specific radioactivity from the aggregation stage to culmination in *D. discoideum*. Cultures prelabeled with [^{14}C]glucose were allowed to differentiate for the specified intervals (see Methods). ⊡ and ■ represent glycogen and protein specific radioactivity, respectively, using the KOH method for glycogen extraction and the trichloroacetic acid method for protein precipitation. In order to obtain glycogen of lower specific radioactivity, cultures were prelabeled with one-tenth of the concentration of glucose and total counts. ⊙ and ● represent glycogen and protein specific radioactivity, respectively, using the trichloroacetic acid method for the isolation of both glycogen and protein.

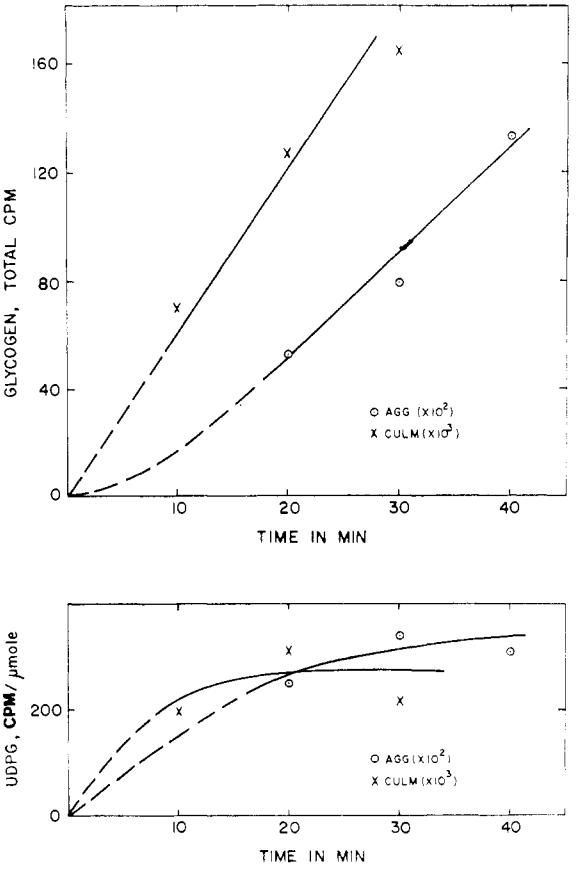

FIGURE 2: Glycogen synthesis at the aggregation and culmination stages of differentiation. Procedures for the isolation of glycogen and UDPG are given in Methods.

TABLE II: Summary of the Rates of Glycogen Synthesis at the Aggregation Stage and at Culmination in *D. discoideum*.

Rate of Glycogen Synthesis (μmoles/min per ml of packed cells)	
Aggregation	Culmination
0.053[a,c]	0.081[a,c]
0.060[c]	0.190[c]
0.027[b]	0.200[b]
0.022	0.240[c]
0.026	0.160
0.013	0.130
	0.150
Mean 0.034 ± 0.019	Mean 0.160 ± 0.052
Range 0.015–0.053	Range 0.108–0.212

[a,b] Stage studies, *i.e.*, cells from the same original culture. [c] Experiments were done in Bonner's-Tris (pH 7.4); all others in Bonner's-MES (pH 6.5).

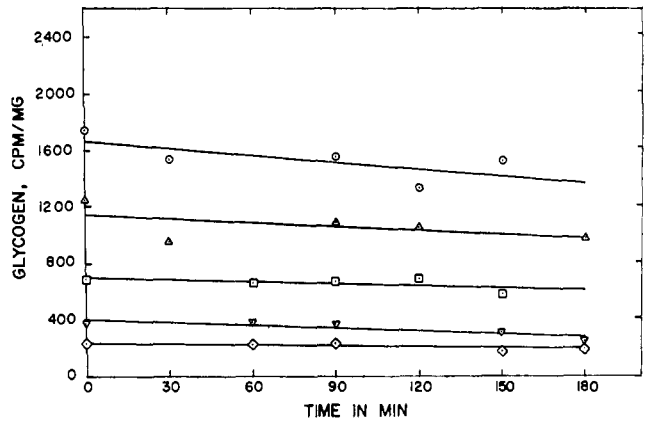

FIGURE 3: Glycogen turnover at aggregation. Data are from five independent experiments at aggregation (see Methods). Each curve was generated from data obtained in a single experiment using five sampling times; each point represents an average of four independent cultures analyzed in parallel. Cultures chased on agar (0.001 M MES, pH 6.5) were ○, initial glycogen extraction with KOH; □, trichloroacetic acid; and ◇, trichloroacetic acid. Cultures chased in liquid were △, Bonner's-MES (pH 6.5) (initial glycogen extraction with trichloroacetic acid) and ▽, Bonner's-Tris (pH 7.4), KOH.

During culmination, it was considered possible that the hypothetically metabolically more active presumptive spore cells would exhibit a higher rate of glycogen synthesis than the presumptive stalk cells. A comparison was therefore made of the rates obtained from simultaneous exposure of both cell types to [^{14}C]glucose and selective exposure of the stalk cells. At culmination, half the cells were incubated with [^{14}C]glucose under the usual conditions (liquid). The other half was differentiating on thin agar layers in petri dishes, and was exposed to a solution of [^{14}C]glucose added beneath the agar, which was momentarily lifted with an imbedded gauze strip. Since the presumptive spore cells were not in direct contact with the agar, the presumptive stalk cells were initially, and probably selectively, exposed to labeled glucose. Under liquid conditions, a value of 0.15 μmole/min per ml of packed cells was obtained; for the companion experiment on agar, this value was 0.23 μmole/min per ml of packed cells. These rates fall within the limits of the values found previously (see Table II).

Chase Experiments. The data obtained in two representative chase experiments are shown in Table III. Cultures which had been prelabeled with [U-^{14}C]glucose and allowed to differentiate to the desired stage were exposed to high concentrations of [^{12}C]glucose. This chase was carried out for the time intervals specified. At least three, and usually four, independent cultures were used for each time interval. The values obtained for each time interval were averaged to give a mean specific radioactivity, which was then plotted as a single point. The data from seven such experiments are shown in Figures 3, 4, and 5. In the regression analyses, however, the individual determinations were used for generating the statistics of the curves in order to increase the precision of the point estimates. Five curves for each stage are given in Figures 3 and 4, and two for each stage in Figure 5. As noted below the figures, two curves for each stage were obtained using the liquid chase technique and the remaining five using the agar chase technique (see Methods). Evidently, there is no difference in the measured rate of glycogen turnover when cells immersed in buffer are used, under our experimental conditions. Both methods for glycogen isolation (KOH and trichloroacetic acid) were employed in obtaining these data and no significant difference in the rate estimates

TABLE III: Data Obtained from Chase Experiments at Aggregation and Culmination Stages.[a]

Chase time (min)	Glycogen Specific Radioactivity (cpm/mg)	Mean
	Aggregation	
0	216, 215, 258, 230	230
60	250, 211, 254, 207	230
90	294, 254, 200, 188	234
150	160, 161, 198, 201	180
180	221, 193, 202, 160	194
	Culmination	
0	605, 590, 620, 555	592
60	525, 545, 540, 525	534
120	397, 370, 405, 384	372
180	294, 296, 290, 281	290

[a] Data obtained using [^{14}C]glucose-labeled cells of *D. discoideum* which were allowed to differentiate at 23° to the desired stage and then exposed to [^{12}C]glucose for specified time periods. The values at each chase time represent separate cultures analyzed independently in parallel. The regression curves generated from these data are shown for aggregation in Figure 3, and for culmination in Figure 5. The variation seen here typified that of each replication of this experiment done at aggregation and culmination (seven per stage).

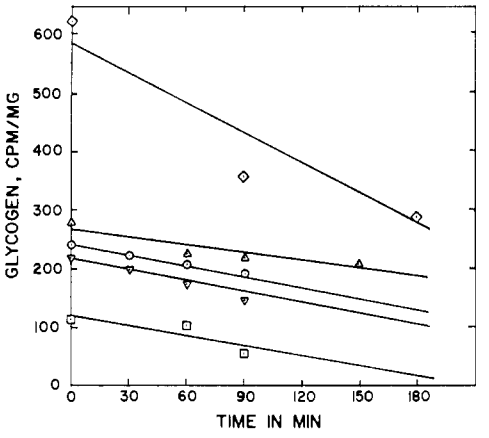

FIGURE 4: Glycogen turnover at culmination. Data are from five independent experiments at culmination (see Methods). Each curve was generated from data obtained in a single experiment; each point represents an average of three or four independent cultures analyzed in parallel. Cultures chased on agar (0.001 M MES, pH 6.5) were ▽, initial glycogen extraction with KOH; □, trichloroacetic acid; ◇, trichloroacetic acid. Cultures chased in liquid were △, Bonner's-Tris, pH 7.4, initial glycogen extraction with trichloroacetic acid; ○, Bonner's-MES, pH 6.5, KOH.

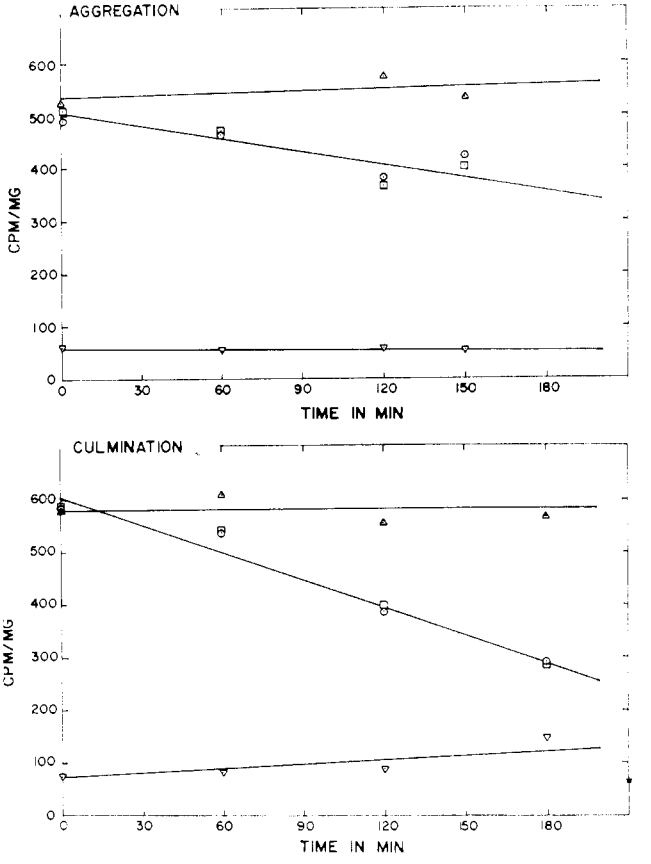

FIGURE 5: Relative specific radioactivities of glycogen and trichloroacetic acid insoluble protein at aggregation and culmination. Prelabeled cells were allowed to differentiate to the desired stage of development (see Methods). Half of the cultures was chased with [^{12}C]glucose and incubation was allowed to proceed at 23° for the periods specified. Each glycogen point represents the average of at least three independent determinations. The specific radioactivity of the trichloroacetic acid insoluble protein is averaged for all the cultures at each sampling time, since no significant differences were found. Nonchased cultures, △; cultures chased with 10^{-2} M, □ or 10^{-3} M, ○ [^{12}C]glucose; trichloroacetic acid insoluble protein, ▽.

resulted. However, the KOH method does not give as great a recovery of glycogen, nor does it permit a determination of both glycogen and protein specific radioactivity from the same culture, since the protein fraction is degraded under these conditions. In the experiments performed using the KOH method for initial glycogen extraction protein specific radioactivities were determined in duplicate from separate cultures harvested in parallel and using the trichloroacetic acid method.

The results of control experiments are shown in Figure 5. Obviously there is no difference in the rate of glycogen turnover found using 10^{-2} or 10^{-3} M glucose for the chase. It is also clear that similar rates were obtained under conditions where pH and buffer were varied. The specific radioactivity of the trichloroacetic acid insoluble protein fraction remained below that of glycogen under our experimental conditions. There were no significant differences in the specific radioactivity of the protein isolated from the series chased with 10^{-2} M glucose, 10^{-3} M glucose, or from the nonchased series at each sampling time. Therefore, the specific radioactivities of the protein at each sampling time were averaged to give the profile of specific radioactivity seen in Figure 5.

TABLE IV: Summary of the Rates of Glycogen Turnover at the Aggregation Stage and at Culmination in *D. discoideum*.

Rate of Glycogen Turnover (μmole/min per ml of packed cells)	
Aggregation	Culmination
0.046	0.173
0.041	0.148
0.036	0.118
0.040	0.099
0.039	0.122
0.059	0.122
0.059	0.122
Mean 0.046 ± 0.01	Mean 0.129 ± 0.02
Range 0.036–0.056	Range 0.109–0.149

[a] These rates were calculated from the data plotted in Figures 3, 4, and 5.

Turnover Rate. Referring again to Figure 5, it can be seen that, under the nonchase conditions, the level of glycogen specific radioactivity remains constant over the interval of the experiment both at aggregation and at culmination. Since the amount of glycogen present in the cells at these stages, *i.e.*, 48.5 μmoles at aggregation and 41.5 μmoles at culmination, also remains constant during these time intervals, calculation of the turnover rate is mathematically straightforward (Reiner, 1953).

Seven independent turnover rates for each stage of differentiation studied are listed in Table IV. These rates were cal-

culated from the curves shown in Figures 3, 4, and 5; for each series, each value is the result of an independent replication of the same experiment consisting of at least three independent determinations at each chase time. For determinations at the aggregation stage of differentiation it was necessary to allow the cells to initiate aggregation before labeling to ensure uptake of a sufficient amount of [^{14}C]glucose.

The difference between the rate of turnover at these two stages of differentiation is significant, *i.e.*, the calculated t statistic is 8.536 while the tabled value at $P \leq 0.01$ (12 degrees of freedom) is 3.055.

Discussion

During a number of the experiments designed to determine the rate of glycogen synthesis, the specific radioactivity of the UDPG pool was somewhat variable. However, the variability encountered in individual experiments did not seriously affect the calculated rate of glycogen synthesis. For example, in the experiment described in Figure 2 at culmination, this rate is 0.16 μmole/min per ml if based on the increase with time in radioactivity of the glycogen pool obtained by subtracting the 10- from the 30-min value, and on the average specific radioactivity of UDPG at 10 and 30 min. Based on the increase in the radioactivity of glycogen between 10 and 20 min, and on the average specific radioactivity of UDPG at 10 and 20 min, this value is 0.14; based on the increase in radioactivity of glycogen obtained from the slope of a line using all three time periods and on the average specific radioactivity of UDPG at 10 and 20 min, this value is 0.13.

Considering the differences in technique and in potential sources of error involved in the determination of glycogen synthesis compared to glycogen turnover, the average values obtained in the two studies are in excellent agreement (Tables II and IV). These values, in turn, are in good agreement with those obtained by Pannbacker (1967) for the rate of UDPG synthesis. Correcting for intercellular water, the latter values are 0.04 and 0.12 μmole/min per ml at aggregation and culmination, respectively. If UDPG is the only direct precursor of glycogen *in vivo*, then its rate of synthesis must be equal to or greater than the rate of glycogen synthesis. The fact that the values are similar is evidence in support of the prediction that UDPG is the real precursor of glycogen in the intact cell and that the synthesis of this polysaccharide is the major fate of UDPG until culmination. If 90% of the UDPG was involved in the synthesis of other carbohydrate materials, glycogen turnover could have been an order of magnitude lower than UDPG turnover; if glycogen were synthesized entirely or in part from glucose *via* a pathway not involving UDPG, glycogen turnover could have been an order of magnitude higher than UDPG turnover.

The observations that the rate of glycogen synthesis equals that of turnover and of UDPG synthesis, and that these values all increase about threefold from aggregation to culmination, substantiate the predictions of our kinetic model. The increased rate of UDPG synthesis is apparently due to greater glucose 1-phosphate availability (Wright *et al.*, 1968), the increased rate of glycogen synthesis to enhanced UDPG and glucose-6-P availability (Wright, 1966), and the increased glycogen degradation to higher levels of glycogen phosphorylase and P_i (Jones and Wright, 1970).

Our results lend further support to the conclusion that data concerning changes in enzyme level *in vitro* frequently have little bearing upon changes of metabolic activity *in vivo* (Wright, 1968). For example, glycogen synthetase activity decreases some tenfold from aggregation to culmination (Wright and Dahlberg, 1967), yet the rate of glycogen synthesis measured *in vivo* actually increases a fewfold during this period. Furthermore, the enzyme activity *in vitro* of glycogen synthetase is only $^1/_{40}$ of that enzyme activity required *in vivo* for the actual rate of glycogen synthesis observed. (The predicted enzyme activity *in vivo* can be calculated from the *in vivo* rate of synthesis, the substrate concentrations, and known K_m values.) These discrepancies indicate that the changes in this enzyme level measured at successive stages during differentiation are not relevant *in vivo*, and may be due to artifacts, such as stage-dependent alterations in enzyme stability during isolation, the relative concentration of activators or inhibitors in extracts, and so forth.

Glycogen phosphorylase, on the other hand, increases at least fivefold from aggregation to culmination; however, the activity of this enzyme (as well as amylase) is fivefold in excess of that required *in vivo* for the actual rate of glycogen degradation observed (Jones and Wright, 1970). The data therefore suggest that these degradative enzymes may be inhibited *in vivo* (a common observation; see, for example, Harris, 1946; Elson, 1958; Chaloupka and Krčkŏvá, 1962) and that this inhibition is released under the *in vitro* assay conditions. Such assays may, therefore, give us very little information concerning the role of enzymes in the intact cell during differentiation.

In the case of UDPG-pyrophosphorylase, although studies *in vitro* indicate an increase in specific activity during differentiation, many other lines of evidence suggest that, in effect, the enzyme does not increase in activity *in vivo*, by even as much as threefold (Wright, 1968). The reasons for this conclusion have apparently escaped the attention of other investigators concerned only with *in vitro* measurements of enzyme activity (Newell and Sussman, 1969). Moreover, data based on experiments with actinomycin D have been interpreted to mean that the message for the synthesis of UDPG-pyrophosphorylase is absent initially, and transcribed during aggregation (Roth *et al.*, 1968). As discussed above, data obtained *in vivo* indicate that, during aggregation, this enzyme catalyzes the rapid production of UDPG at a rate comparable to the rate of synthesis of soluble glycogen, of which it is the precursor. Theoretical analyses have been carried out to examine the effects of a tenfold increase in the concentration of UDPG-pyrophosphorylase on the rate of UDPG synthesis and on the steady state pool levels of glucose-1-P and UDPG (Wright, 1970). The effects observed were the same, regardless of the s/K_m ratio, *i.e.*, this ratio is not a critical variable for an enzyme in this kinetic position, over the time periods involved and under the steady-state conditions of the living cell. Thus, the absolute level of metabolities, which may be affected by compartmentalization, is relatively unimportant in this case. An important parameter, however, concerns *changes* in metabolite concentration, an observation also made recently by Rothman and Cabib (1969) in connection with the regulation of glycogen synthesis in intact yeast cells. Data on the relative concentration of metabolites, obtained by killing cells immediately with acid or heat, are likely to be more reliable than comparable data on enzymes, which are intrinsically more labile and must also be isolated and assayed

under conditions permitting continued changes in enzymatic activity.

That the glucose of the glycogen pool is not being diverted significantly to protein (or *vice versa*) has been established by showing that the specific radioactivity of the protein remains relatively constant and below that of glycogen (Figure 2). If there were active gluconeogenesis, the specific radioactivity of glycogen would decrease with time due to an intracellular "chase." That this did not occur allows estimation of the rate of glycogen turnover without correction due to this potential source of [^{12}C]glucose. The relative unimportance of gluconeogenesis in *D. discoideum* has been discussed previously (Baumann and Wright, 1968; Cleland and Coe, 1968). The fact that the specific radioactivity of the glycogen pool is constant supports an assumption of the kinetic model, namely, that glucose units of this pool are being recycled until the culmination stage of differentiation.

The observation that the rates of synthesis and turnover equal one another at both aggregation and culmination would be expected from the fact that the glycogen level remains essentially constant during this period. For our purposes, glycogen turnover will be defined as the rate of replacement of glucose units in the glycogen pool; this was the primary purpose of these investigations, rather than to obtain information about the turnover of glycogen molecules *per se*. Since glucose units are recycled and not lost from the pool, calculation of the rate of glucose unit turnover is greatly simplified. It need only be assumed that the fraction of the glycogen pool labeled by [^{14}C]glucose is the same as that chased by [^{12}C]glucose. That this assumption is valid is indicated by the close agreement in the observed rates of turnover and synthesis. Assumptions regarding the metabolic homogeneity of glycogen in this system do not bear directly upon the flux values determined, although information concerning this point has been obtained.

In mammalian systems, it has been found that during relatively short periods following a single exposure to [^{14}C]-glucose, glycogen is labeled predominantly in peripheral glucose residues; with time, the label becomes centrally located (for a review, see Stetten and Stetten, 1960). Therefore, the nonreducing, peripheral glucose residues are metabolically the most reactive, a conclusion in accordance with enzymological data concerning the initial entry and subsequent redistribution of glucose residues in glycogen (Larner *et al.*, 1952; Larner, 1953). In the present investigation, the agreement between the rates of synthesis and turnover suggests that peripherally located glucose residues are predominantly involved in both studies. This would be expected in the case of glycogen synthesis, which was determined over periods of less than 1 hr (Figure 1). The procedure for determining the rate of glycogen turnover, however, involved much longer time periods with respect to (a) the time between the initial labeling of the glycogen pool and the subsequent chase and (b) the actual time of the chase (about 3 hr; see Figure 3). Had labeled glucose residues been redistributed to less metabolically reactive positions during this time, they would not be readily replaced during the chase, and the observed rate of turnover would have been significantly lower than the rate of synthesis. In fact, the mean values at culmination for turnover and synthesis do indicate a tendency in this direction. The fact that the percentage of counts released on β-amylase digestion was twice the percentage of sugar released also indicates peripheral labeling.

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