# Uridine Diphosphoglucose Biosynthesis during Differentiation in the Cellular Slime Mold. I. *In Vivo* Measurements*

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ABSTRACT: Following incorporation of [$^{14}$C]uracil into cells of *Dictyostelium discoideum*, the rate of [$^{14}$C]uridine diphosphoglucose ([$^{14}$C]UDPG) pool replacement (by material of the specific radioactivity of endogenous [$^{14}$C]uridine 5′-triphosphate) was determined at two stages of differentiation. The UDPG pool size increased 2.2-fold between the two stages, whereas the rate of pool replacement increased only 1.6-fold. This indicated that the rate of synthesis was higher at the second stage but that no major increase in the net capacity for utilization of UDPG had occurred.

Accumulation of an alkali-insoluble carbohydrate (AIC)[1] is a biochemical process specifically associated with the terminal stages of development in the cellular slime mold (the stage of sorocarp formation, "culmination," during which irreversible differentiation occurs). Ward and Wright (1965) have examined an enzyme which synthesizes AIC (a complex of glycogen and cellulose) from UDPG, and find that the $K_m$ of this enzyme is higher than the intracellular levels of UDPG found in the cell. As UDPG levels are known to rise prior to the accumulation of AIC (Wright *et al.*, 1964), it would seem that increased UDPG availability contributes to the accumulation of AIC at the culmination stages. A similar situation may hold for two other carbohydrates (trehalose and a mucopolysaccharide) which also accumulate at this time and are synthesized in part from UDPG (Ceccarini, 1965; Roth and Sussman, 1966; Sussman and Osborn, 1964). This paper describes measurements of the rate *in vivo* of UDPG pool replacement, obtained by following incorporation of radioactivity into cellular UTP and UDPG pools during incubation with [$^{14}$C]uracil. From these observations calculations are made of the fraction of the UDPG pool replaced per minute by material of the specific radioactivity of the UTP pool.

During these incubations with [$^{14}$C]uracil, pool sizes did not change, *i.e.*, the pools were in a steady state, in which rate of synthesis equalled rate of utilization. Between the aggregation and culmination states of development, UDPG pool size increased; thus a different steady state existed at the second stage. Little change in UDPG pool replacement rate occurred between these stages, indicating that rates of UDPG synthesis and utilization increased at the second stage in the absence of a major enhancement in the cell's enzymatic capacity to utilize UDPG.

## Materials and Methods

*Materials.* UTP and UDPG were obtained from Sigma Chemical Co., St. Louis. [1-$^{14}$C]Glucose and [$^{14}$C]uracil were purchased from New England Nuclear Corp., Boston, Mass. [$^{14}$C]Uracil was also obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Darco G-60 activated charcoal (Atlas Powder Co., Wilmington, Del.) was acid washed before use by suspending in a large volume of 3 M HCl, bringing the suspension to a boil, then allowing the charcoal to settle and decanting the supernatant (plus fines). This process was repeated once with acid, then with glass-distilled water until the washings no longer formed a precipitate with saturated silver nitrate. The charcoal was then dried overnight at 110°.

PEI-cellulose thin layer chromatograms were prepared according to Randerath and Randerath (1966). PEI (polymerized ethylenimine) was the kind gift of Chemirad Corp., East Brunswick, N. J. MN-300 cellulose was manufactured by Macherey, Nagel and Co., West Germany. The thin layer was spread on Bakelite rigid vinyl sheets manufactured by Union Carbide Corp., Cincinnati, Ohio.

*Growth of Cells. Dictyostelium discoideum* strain NC-4 was grown on a complex medium in the presence of *Escherichia coli* and harvested as previously described (Liddel and Wright, 1961). The cells were then allowed to develop on 2% agar 0.01 M in phosphate buffer (pH 6.5) and 0.001 M in EDTA. On this medium differentiation of cell aggregates is very synchronous. "Aggregation" occurs at ~12 hr and during the next 12 hr migrating pseudoplasmodia appeared which, at

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[1] Abbreviations used: AIC, alkali-insoluble carbohydrate; UDPG, uridine diphosphoglucose; UTP, uridine 5′-triphosphate.

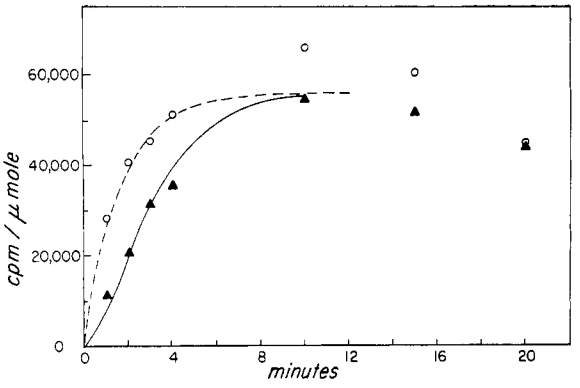


FIGURE 1: Incorporation of radioactivity into UTP and UDPG during incubation with [$^{14}$C]uracil: (open circles) UTP specific radioactivity; (closed triangles) UDPG specific radioactivity. The curves are calculated according to equations presented in the text.

the end of this period, began formation of the sorocarp ("culmination stage"). The cells were incubated at 23°.

*Incubation with [$^{14}$C]Uracil.* Cells at a desired stage of development were rinsed off plain agar in Bonner's (1947) standard solution ($10^{-3}$ M in pH 7.4 Tris buffer) and 5-ml portions (approximately 0.5-ml packed cell volume, or 30 mg of dry weight of cells) were incubated at 23° with gentle swirling in 50-ml erlenmeyer flasks. The volume of packed cells was determined by centrifuging an aliquot of the suspension medium at 1000*g* for 10 min. At zero time 0.038 μmole of [$^{14}$C]uracil (40 mc/mmole) was added to each flask. This level of exogenous uracil was shown in another experiment not to affect developmental rate or the composition of the acid-soluble nucleotide pool. Following exposure to [$^{14}$C]uracil for the prescribed times (less than 30 min, during which pool level and cell volume did not change) an equal volume of cold 0.70 M $HClO_4$ was added to each flask. Extraction with acid was allowed to proceed for 30 min at 4° with occasional stirring. The suspension was centrifuged, the pellet was washed with 2 ml of 0.35 M $HClO_4$ and centrifuged, and the combined supernatants were neutralized with 5 N KOH. $KClO_4$ was removed by centrifugation.

*Adsorption on Acid-Washed Charcoal.* The $A_{260}$ of the neutralized extract was adjusted to ~2 and the material absorbing light at this wavelength was completely adsorbed by the addition of 15 mg of acid-washed charcoal/ml. The charcoal was collected on a Millipore filter, washed with an equal volume of glass-distilled water, and then eluted with three volumes of cold ammoniacal ethanol (2:50:50, $NH_4OH$–ETOH–$H_2O$). This procedure quantitatively recovered the material absorbing light at 260 mμ as well as added authentic nucleotides (Pannbacker, 1967). The eluate was reduced in volume by evaporation under vacuum (until most of the ethanol was removed), followed by lyophilization (Thermovac Portable Freeze-Dryer, Thermovac Industries Corp., Brooklyn, N. Y.).

*Thin Layer Chromatography.* The concentrated eluate was applied to PEI-cellulose thin layer chromatograms. UTP was isolated by two-dimensional chromatography using a stepwise LiCl development in the first dimension and stepwise formate buffer (pH 3.4) in the second (Randerath and Randerath, 1964). UDPG was isolated by developing in the first dimension in an acetic acid–LiCl mixture, and in the second with borate buffer (Randerath and Randerath, 1965). Spots corresponding to UTP or UDPG were cut out of the plastic-backed sheets and eluted for 1 hr in 1 ml of 0.7 M $MgCl_2$ (0.02 M in pH 7.4 Tris) at room temperature. This procedure resulted in eluates with a negligible background $A_{260}$ (from the thin layer itself, see Pannbacker, 1967). Quantitative recovery of authentic nucleotides was obtained by these methods; indeed, 80% of the original $A_{260}$ of the extract could be accounted for as identifiable nucleotides.

The specific radioactivity of the eluates (UTP and UDPG) was determined by counting 0.5 ml of the eluate in 10 ml of liquid scintillation gel using a Packard Tri-Carb liquid scintillation counter, Model 314-DC, and relating the radioactivity measured to the $A_{260}$ of the eluate. Internal quenching of scintillation was checked by the channel ratio method (no appreciable quenching was encountered). Variability in counter efficiency was compensated for by always counting precursor (UTP) and product (UDPG) samples at the same time.

## Results and Discussion

*Calculation of Rates of Incorporation of [$^{14}$C]Uracil.* Figure 1 shows the course of appearance of radioactivity in UTP and UDPG during exposure of cells to [$^{14}$C]uracil. The specific radioactivity (SA) of UTP rises immediately and approaches a maximum in an exponential manner, allowing one to describe the curve as

$$SA_{UTP} = C(1 - e^{-k_1 t}) \text{ cpm}/\mu\text{mole} \qquad (1)$$

in which $SA_{UTP}$ is the specific radioactivity of the UTP pool at time $t$, $C$ is the maximum specific radioactivity, and $k_1$ is the rate (reciprocal minutes) at which the UTP pool is replaced by material of specific radioactivity = $C$. Since the radioactivity in UDPG comes from UTP, the course of its appearance can be described as

$$SA_{UDPG} = C[((1 - k_2)/(k_2 - k_1)e^{-k_1 t}) - ((1 - k_2)/(k_2 - k_1)e^{-k_2 t})] \text{ cpm}/\mu\text{mole} \qquad (2)$$

in which $SA_{UDPG}$ is the specific radioactivity of the UDPG pool at time $t$, and $k_2$ is the rate (reciprocal minutes) at which the UDPG pool is replaced by material of $SA_{UTP}$ (equations similar to this have been previously derived; for example, see Roberts *et al.*, 1955). Use of this expression requires that pre-

cursor and product pool sizes do not change during the course of the experiment. This has been verified for UTP and UDPG in each case.

In Figure 1, the best fit to the early UTP points was obtained by estimating $C$ from the apparent saturation value of the curve (55,600 cpm/$\mu$mole) and trying several values of $k_1$ according to eq 1 (all early points fell between curves corresponding to $k_1$ = 0.50–0.70). A value of 0.60 $min^{-1}$ for $k_1$ gave the curve indicated by the dashed line. Using these values, various values were tried for $k_2$ in eq 2. It was found that all early points fell between curves corresponding to $k_2$ values of 0.50–0.75 $min^{-1}$; 0.65 $min^{-1}$ gave the curve described by the solid line in Figure 1.

*Pool Replacement and UDPG Synthesis.* In eq 2, $k_2$ is the rate of UDPG pool replacement, determined from the rate at which equilibration was obtained between the UDPG and UTP pools. Multiplying this quantity by the UDPG pool size ($S$) (see Methods) gives the rate at which UDPG is synthesized ($v_{syn}$): $v_{syn} = k_2S$ $\mu$mole/min ml. Table I summarizes results of several experiments in which pool replacement, pool size, and rate of synthesis were determined. In the last four examples, aggregation and culmination stages are compared. The average increase in pool size between these stages is seen to be 2.2-fold while the rate of synthesis increases on the average 3.5-fold. This indicates that a higher rate of UDPG synthesis contributes to the greater steady-state pool size at the second stage.

*Utilization of UDPG.* A quantitative statement about the kinetics of UDPG utilization can be made if one assumes that the level of UDPG is well below the $K_m$ of any enzyme utilizing it. This is known to be the case in the synthesis of both soluble glycogen and cell wall glycogen (Ward and Wright, 1965; Wright, 1966). In fact, the $K_m$'s of all enzymes so far studied in the slime mold have been found to be higher than the levels *in vivo* of the substrates involved (Wright, 1966). This is in agreement with data from mammalian systems as reported by Vegotsky and Frieden (1958).

The Michaelis equation relating velocity to substrate concentration ($v = V/(1 + K_m/S)$) can be approximated by ($v = V/K_mS$) in the case in which $S$ is much smaller than $K_m$. Thus the velocity of utilization of UDPG ($v_{utilzn}$) can be approximated by the expression

$$v_{utilzn} = \left[\frac{V_1}{K_1} + \frac{V_2}{K_2}; \cdots\cdots \frac{V_n}{K_n}\right]S$$

in which $n$ reactions utilize UDPG.

It was stated earlier that, during an incubation, pool size does not change; thus $v_{syn} = v_{utilzn}$. It follows from eq 3 that $v_{utilzn}$ is also equal to $k_2S$, so

$$k_2 = \left[\frac{V_1}{K_1} + \frac{V_2}{K_2} \cdots \frac{V_n}{K_n}\right]$$

This means that, in a situation in which the level of substrate is below the $K_m$'s of all reactions which utilize it, the rate of substrate pool replacement gives a direct measurement of what may be called the "enzymatic capacity" for the substrate.

In Table I the column on the extreme right shows the ratio of $k_2$ at aggregation and culmination. The rate of pool replacement increases an average of 1.6 times. This increase may reflect complex changes in the enzymes utilizing UDPG (loss of some enzymes, gain of others); however, one observation suggests that enzymes utilizing UDPG are not lost between stages. The presumed major drain on UDPG at aggregation (soluble glycogen synthetase) is still present at high levels at the culmination stage (insoluble cell wall glycogen synthetase). A fraction of the enzyme which synthesizes soluble glycogen throughout differentiation begins to synthesize AIC at the later stages (Wright *et al.*, 1966; Wright, 1966). If this is typical, there is not as large an increase in enzymatic capacity for UDPG as might at first be expected from the observation that three carbohydrates accumulate only at the later stage (AIC, trehalose, and a mucopolysaccharide). One way in which a new product may arise without the appearance of a new enzyme activity is for existing enzymes to utilize a different primer. This seems to be the case in the synthesis of AIC. Another mechanism by which accumulation of a product may be brought about without changing the activity of enzymes synthesizing it is to decrease the rate of

TABLE I: UDPG Pool Size, Rate of Replacement, and Synthesis during Development.

| Expt | Stage[a] | UDPG Pool Replacement (%/min)[b] | UDPG Pool Size (M × $10^{-4}$) | UDPG Synthesis ($\mu$moles/min)/ml of pcv[c] | $k_2$ Culm/$k_2$ Agg |
|---|---|---|---|---|---|
| A | Preculm | 20 | 1.29 | 0.026 | |
| | Culm | 10 | 1.95 | 0.020 | |
| B | Pseudo | 20 | 1.11 | 0.022 | |
| | Preculm | 20 | 1.19 | 0.024 | |
| | Culm | 20 | 1.28 | 0.026 | |
| C | Culm | 25 | 3.95 | 0.099 | |
| D | Agg | 50 | 1.28 | 0.064 | |
| | Culm | 65 | 2.08 | 0.135 | 1.3 |
| E | Agg | 20 | 1.31 | 0.026 | |
| | Culm | 50 | 3.57 | 0.179 | 2.5 |
| F | Agg | 20 | 0.50 | 0.010 | |
| | Culm | 20 | 1.63 | 0.033 | 1.0 |
| G | Agg | 10 | 1.51 | 0.015 | |
| | Culm | 15 | 2.04 | 0.031 | 1.5 |

[a] Agg = aggregation; preculm = preculmination; culm = culmination; pseudo = pseudoplasmodium. [b] ($= K_2 \times 100$). [c] pcv = packed cell volume.

product breakdown. This may be involved in trehalose accumulation, since it is known that some trehalose is present at all stages and trehalose activity falls at culmination (Ceccarini, 1965; Roth and Sussman, 1966). The utilization of UDPG for mucopolysaccharide synthesis presumably occurs by way of the epimerase reaction (the mucopolysaccharide is composed of galactose derivatives (White and Sussman, 1963)). It has been observed by H. Wu[2] that epimerase levels do not change significantly during development in this organism.

*Alkali-Insoluble Carbohydrate Synthesis in Vivo.* Table II presents the results of an experiment in which incorporation of radioactivity from [1-$^{14}$C]glucose into AIC was measured in order to determine the rate of AIC synthesis *in vivo*. Cells were incubated with [1-$^{14}$C]-glucose (0.1 μmole/flask), and UTP and UDPG extracted and isolated as in the experiments with [$^{14}$C]-uracil. AIC was isolated from the acid-insoluble pellet according to Ward and Wright (1965). UDPG was the only nucleotide sugar labeled significantly with [1-$^{14}$C]-glucose as shown by autoradiography of the thin layer chromatogram (see Pannbacker, 1967). A smaller spot corresponding in $R_F$ to UDPGal was also labeled. The UTP isolated from these preparations contained no radioactivity, indicating that all radioactivity present in the UDPG was in the glucose moiety.

TABLE II: Alkali-Insoluble Carbohydrate Synthesis *in Vivo*.

| Time (min) | Cpm in UDPG Pool[a] | Cpm/μmole of UDPG | Cpm in Alkali-Insoluble Carbohydrate[a] |
|---|---|---|---|
| 0 | 0 | 0 | 0 |
| 10 | 3,460 | 13,400 | 4,100 |
| 20 | 3,550 | 10,300 | 7,300 |

[a] Total counts in each sample of volume = 0.84 ml of packed cells.

In this experiment, radioactivity appeared in the alkali-insoluble fraction at a rate of about 365 cpm/min. The average specific radioactivity of the UDPG pool was 11,800 cpm/μmole; thus 0.031 μmole of glucose was incorporated/min per 0.84 ml of packed cells, or 0.03/μmole per min per ml of packed cells.

AIC accumulates to a level of about 20 μmoles/ml of packed cells (5% dry weight) in about 10 hr. This is a rate of appearance of 0.033 μmole/min per ml, which agrees well with the rate of incorporation of radioactive glucose. The rate of UDPG synthesis was measured on the same batch of cells in a parallel experiment using [$^{14}$C]uracil (expt C of Table I), and was estimated to be 0.099 μmole/min per ml. This value (three times the rate of AIC synthesis) is consistent with the predicted demand for UDPG; two other carbohydrate fractions, trehalose and a mucopolysaccharide, are also accumulating at this time and to approximately the same extent as does AIC.

[2] Personal communication.

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