Uridine Diphosphoglucose Biosynthesis during Differentiation in the Cellular Slime Mold. II. *In Vitro* Measurements*

Richard G. Pannbacker†

ABSTRACT: Factors contributing to the observed increase in rate of uridine diphosphoglucose (UDPG) synthesis between the aggregation and culmination stages of development in the cellular slime mold have been investigated. Comparison of substrate (uridine 5′-triphosphate and glucose 1-phosphate) levels *in vivo* to the K_m's of purified UDPG synthetase (106 times) indicated that increased substrate availability at culmination could account for the increased rate of synthesis of UDPG. The small increase in enzyme level observed between the two stages could contribute to the rate increase *in vivo*, but could also be an artifact due to differential enzyme stability in extracts prepared at the two stages of differentiation.

The study of the metabolism of differentiation has, in large part, been concerned with measurement of changes in enzyme levels. The enzyme which synthesizes UDPG[1] (UDPG pyrophosphorylase, EC 2.7.7.9, UTP:α-D-glucose 1-phosphate uridyltransferase) (Kalckar and Cutolo, 1952; Munch-Petersen *et al.*, 1953) appears to increase in level between the aggregation and culmination stages of morphogenesis in the cellular slime mold. An increase in the rate of UDPG synthesis *in vivo* is also observed between these two stages (see preceding paper). This report examines factors which could contribute to this rate increase *in vivo* and evaluates the relative importance of changes at the enzyme and substrate level.

Materials

Nucleotides were obtained from the Sigma Chemical Co., St. Louis, Mo., and Pabst Laboratories, Inc., Milwaukee, Wis. Sugar phosphates were purchased from Sigma. Streptomycin sulfate was obtained from Parke Davis and Co., Detroit, Mich. Phosphoglucomutase (from rabbit skeletal muscle) and glucose 6-phosphate dehydrogenase (from yeast) were obtained from Calbiochem, Los Angeles, Calif. UDPG dehydrogenase (from bovine liver) was purchased from Sigma.

Methods

Growth of Cells. Dictyostelium discoideum was grown, harvested, and allowed to differentiate on nonnutrient agar as described previously (Liddel and Wright, 1961; see preceding paper).

Enzyme Assays. ASSAY MIXTURE I. For the assay of UDPG pyrophosphorylase in the direction of G-1-P formation from UDPG, the method of Munch-Peterson (1955) was used. Into a quartz microcell of 1-cm light path was pipetted 0.575 ml of a solution 4×10^{-2} M in pH 8.5 Tris buffer, 1.2×10^{-2} M in $MgCl_2$, 2×10^{-3} M in cysteine (freshly neutralized), and 1×10^{-3} M in TPN. To this was added 10 μl of extract, 5 μl of glucose 6-phosphate dehydrogenase, 160 enzyme units/ml,[2] 10 μl of phosphoglucomutase, 20 enzyme units[2]/ml, and 10 μl of 0.1 M UDPG. The reaction was started by the addition of 10 μl of 0.1 M inorganic pyrophosphate, and the increase in A_{340} followed either on a recording instrument (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) or by taking readings each minute on a Zeiss spectrophotometer, Model PMQ II. The increase in A_{340} was linear with time for at least 5 min.

ASSAY MIXTURE II. For the assay of UDPG pyrophosphorylase in the direction of UDPG formation, appearance of UDPG was detected with the specified UDPG dehydrogenase. Into a quartz microcell was pipetted 0.63 ml of a solution 4×10^{-2} M in pH 8.5 Tris buffer and 2×10^{-3} M in cysteine (freshly neutral-

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† Present address: Department of Pharmacology, Washington University School of Medicine, St. Louis, Mo. 63110.

[1] Abbreviations used: UDPG, uridine diphosphoglucose; G-1-P, glucose 1-phosphate; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; UTP, uridine 5′-triphosphate; TDPG, thymidine diphosphoglucose; GDPG, guanosine diphosphoglucose; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GMP, guanosine 5′-monophosphate; UDP, uridine 5′-diphosphate; UMP, uridine 5′-monophosphate; IMP, inosine 5′-monophosphate; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; DAAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; R-5-P, ribose 5-phosphate.

[2] 1 unit = 1 μmole/min.

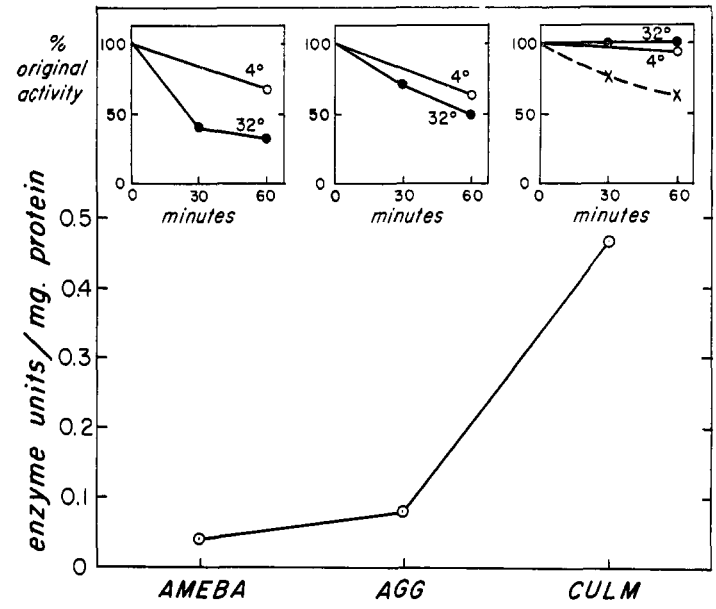

FIGURE 1: Specific activity and stability of UDPG pyrophosphorylase isolated at three stages of development in the absence of UTP. See text for details.

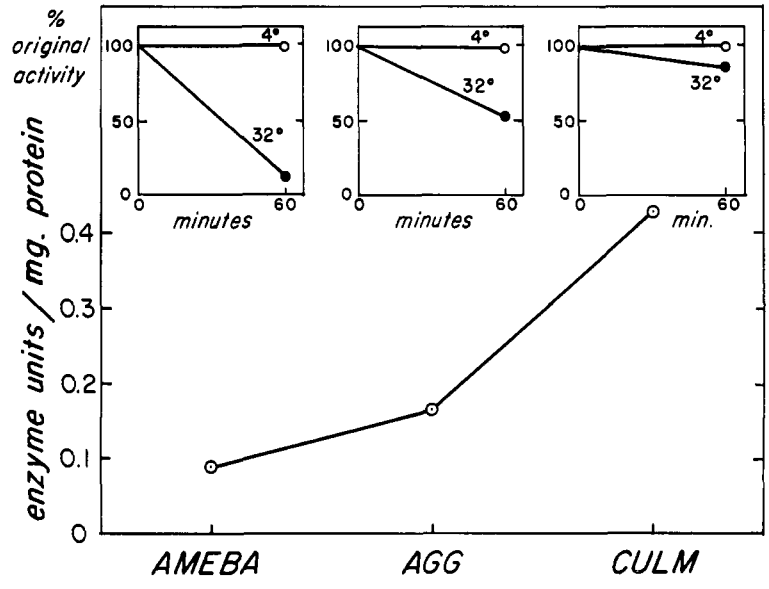

FIGURE 2: Specific activity and stability of UDPG pyrophosphorylase isolated at three stages of development in the presence of UTP. See text for details.

ized). To this was added 10 μl of extract, 10 μl of UDPG dehydrogenase (10,000 units/ml), 10 μl of 5×10^{-2} M DPN, and 10 μl each of UTP and G-1-P (to give the final concentration of substrate desired). $MgCl_2$ was added in equimolar quantities with UTP. The reaction was started by addition of the G-1-P and the increase in A_{340} followed as before.

Preparation of Sephadex G-200 Column. Sephadex G-200 (purchased from Pharmacia Fine Chemicals, Piscataway, N. J.) (2 g) was suspended in 250 ml of 0.01 M Tris buffer (pH 7.4) and allowed to swell for 48 hr. Fines were decanted and the gel was resuspended in 100 ml of buffer and poured into a large funnel (connected to a buffer-filled column 1.12 cm in diameter) and stirred continuously. Flow through the column was not started until the gel had settled overnight, and was then allowed to proceed at a rate of 4 ml/hr for 24 hr. The final column height was 60 cm.

Results

Enzyme Level and Stability during Development. Figure 1 shows the specific activity of UDPG pyrophosphorylase extracted from cells at three stages of development. The cells were suspended in 0.01 M Tris buffer (pH 7.4) frozen in a Dry-Ice–ethanol bath, thawed, and centrifuged at 36,000*g* for 30 min in the refrigerated (2.5°) centrifuge. The boxes above each point show the change in enzyme activity during incubation of these extracts at 4 (open circles) and 32° (closed circles). Extracts from early stages are obviously unstable under these conditions. (All extracts measured were stable during assay; 5 min at 23°, assay mixture I.) The dashed line in the box above the culmination point represents values obtained when a mixture of aggregation and culmination extracts was incubated at 32°. The loss of activity is greater than predicted from losses in extracts incubated separately; thus there seems to be an agent present in early extracts which destroys enzymatic activity. This is a general phenomenon (Wright, 1960), and must be considered in interpreting the inhibition by actinomycin D of UDPG pyrophosphorylase during development (Wright and Pannbacker, 1967).

It has been observed (Wright, 1964) that UTP stabilizes this enzyme. Figure 2 illustrates corresponding data for the same culture in which extraction was carried out in the presence of 0.01 M UTP. Although the enzyme in these extracts seems to be stable at 4°, it is not completely protected by UTP; activity is still lost in extracts of early stages when these are incubated at 32° (Figure 2, closed circles). This suggests that enzyme specific activities obtained at early stages are minimal values; a part of the original activity may be lost by the time extraction is completed.

The apparent increase in enzyme specific activity between the aggregation and culmination stages is about fivefold when enzyme is extracted in the absence of UTP and is ~2.5-fold when extraction is performed in the presence of 0.01 M UTP. Presumably a still smaller difference would be observed if enzyme were completely stabilized during extraction at both stages.

Purification of UDPG Pyrophosphorylase. Slime mold cells were rinsed off plain agar in a cold solution of UTP (0.01 M) and Tris (0.01 M, pH 7.4) and immediately frozen by immersing the tube containing the suspension in a Dry Ice–ethanol bath. The frozen suspension was allowed to thaw and was then centrifuged at 36,000*g* for 30 min in the refrigerated centrifuge. All subsequent procedures were carried out at 4°. Streptomycin sulfate (0.8 ml of 0.4 g/ml) was added slowly, with constant stirring, to 20 ml of the supernatant. The resultant suspension was stirred for an additional 30 min and centrifuged at 12,000*g* for 10 min. The precipitate was discarded. Saturated ammonium sulfate solution at 4° (0.01 M in Tris, pH 7.4) was added slowly to the supernatant with constant stirring until the mixture was 40% saturated. The suspension was stirred for an additional 30 min and centrifuged. The pellet was dissolved in 2 ml of 0.01 M Tris. The

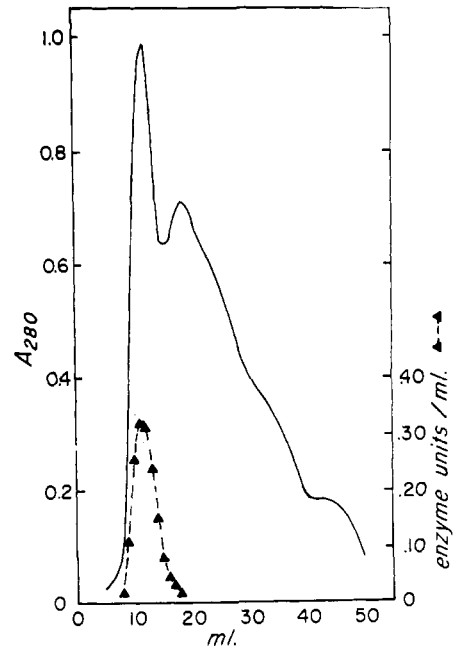

FIGURE 3: Elution profile of protein (A_{280}) and enzyme activity from gel filtration column. See text for column preparation and enzyme assay (assay mixture I).

fractionation was continued in this manner and fractions precipitating in 40–50 and 50–55% saturated ammonium sulfate were collected and their specific activities measured. The material precipitating between 50 and 55% saturation was found to have the highest specific activity (all assays were performed in assay mixture I) and this fraction was applied to the top of a Sephadex G-200 column (1.12 × 60 cm) and eluted with 0.01 M Tris (pH 7.4) at a rate of ~3 ml/min; 1.5-ml fractions were collected. A typical elution profile is shown in Figure 3. The fractions containing enzyme of the highest specific activity were pooled and made 0.05 M in Tris (pH 8.5). Saturated ammonium sulfate solution (0.05 M in pH 8.5 Tris) was added to the combined fractions as before, and fractions precipitating at 0–40, 40–50, 50–55, 55–60, and 60–70% were collected, each being taken up in 1 ml of 0.01 M Tris (pH 7.4). The fraction precipitating between 55 and 60% saturation contained most of the enzyme activity, and the degree of purification obtained was 106-fold over the initial extract, with a yield of 21.4%. A summary of this purification procedure is presented in Table I.

Enzyme was purified from several preparations at both aggregation and culmination stages with essentially the same results. Although the enzyme is stable at 4° both in the crude extract (in the presence of UTP) and in the purified form, the intermediate stages of purification are rather unstable, requiring that the purification be carried out as rapidly as possible.

Characteristics of the Enzymatic Reaction. When measured in either direction, the appearance of product

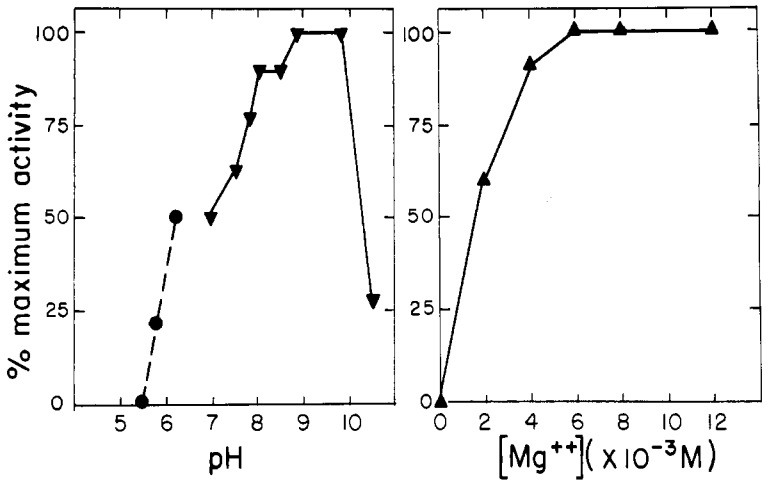

FIGURE 4: The relative rate of appearance of TPNH in assay mixture I as pH of the buffer was varied (a) and the response of reaction velocity to $MgCl_2$ concentration (b). (a) (left) Triangles, Tris buffer; closed circles, acetate buffer. A purified preparation of UDPG pyrophosphorylase was used; assay enzymes were in excess. (b) (right) Measured in the direction of UDPG breakdown in the presence of 4×10^{-3} M PP_i (assay mixture I, see text).

TABLE I: Purification of UDPG Pyrophosphorylase from *D. discoideum*.

Description	Vol. (ml)	Total[a] Protein (mg)	Total Act. (units[b])	Units/ mg	% Yield	Purifcn
Initial extract	20	326	5.20	0.0160	(100)	(1)
Streptomycin·SO_4	20	238	5.20	0.0218	100	1.36
Ammonium sulfate, pH 7.4, 50–55% satd	2.0	18.4	2.40	0.130	46.2	8.1
Sephadex G-200	6.5	2.13	1.17	0.550	22.5	34.4
Ammonium sulfate, pH 8.5, 55–60% satd	1.0	0.656	1.11	1.691	21.4	106

[a] By the Lowry method (Lowry *et al.*, 1951). [b] 1 unit = 1 μmole/min of TPNH produced in assay mixture I (see Methods).

was a linear function of both time and enzyme concentration. The reaction was completely dependent on UDPG and PP_i when measured in the direction of UDPG utilization and on U-T-P and G-1-P when measured in the direction of UDPG synthesis. The pH optimum in the direction of UDPG utilization (for the system of assay mixture I) fell between 8.5 and 10 (see Figure 4a). Mg^{2+} was required for the reaction to proceed in either direction (Villar-Palasi and Larner, 1960). Maximal velocity in the direction of UDPG utilization was attained at a level of 6×10^{-3} M $MgCl_2$ in the presence of 4×10^{-3} M PP_i (see Figure 4b). UDPG pyrophosphorylase from other sources shows a similar requirement (Munch-Peterson, 1955; Ginsburg, 1958). In the direction of UDPG synthesis, the Mg^{2+} requirement was satisfied by adding it in equimolar quantities with UTP. Cysteine stimulated the reaction maximally at a concentration of 1.5×10^{-4} M or above and was routinely included in assay mixtures (Munch-Peterson, 1955). Purified enzyme preparations did not catalyze G-1-P formation from TDPG or GDPG. Crude extracts showed only slight activity toward these compounds.

Michaelis Constants for UTP and G-1-P. Lineweaver–Burk (1934) plots of the reciprocal of reaction velocity *vs.* the reciprocal of substrate concentration are shown in Figure 5 for UTP and Figure 6 for G-1-P. The K_m for the substrate is the negative reciprocal of the intercept on the abscissa in these plots. Purified enzyme preparations were used. As Figure 5 shows, the K_m for UTP is approximately 8×10^{-4} M and is not influenced by the concentration of G-1-P. The data of Figure 6 indicate that the K_m for G-1-P is influenced to some extent by the UTP concentration; at low levels of UTP (1.43×10^{-4} M) the value for the K_m for G-1-P is 2.6×10^{-4} M; at saturating UTP levels it falls to 1.6×10^{-4} M. Similar results are obtained with purified enzyme from both aggregation and culmination stages.

Effects of Other Compounds. To test for possible effectors of this enzyme, 25 compounds were added individually at 10^{-3} M to assay mixture II containing

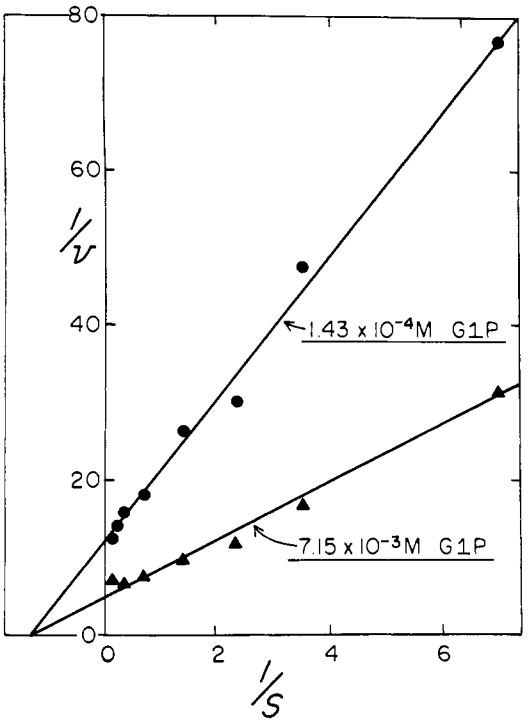

FIGURE 5: Lineweaver–Burk (1934) plot of the response of reaction velocity (arbitrary units) to changes in UTP concentration 10^3 M^{-1} at two levelsof G-1-P. (See text for details, assay mixture II.)

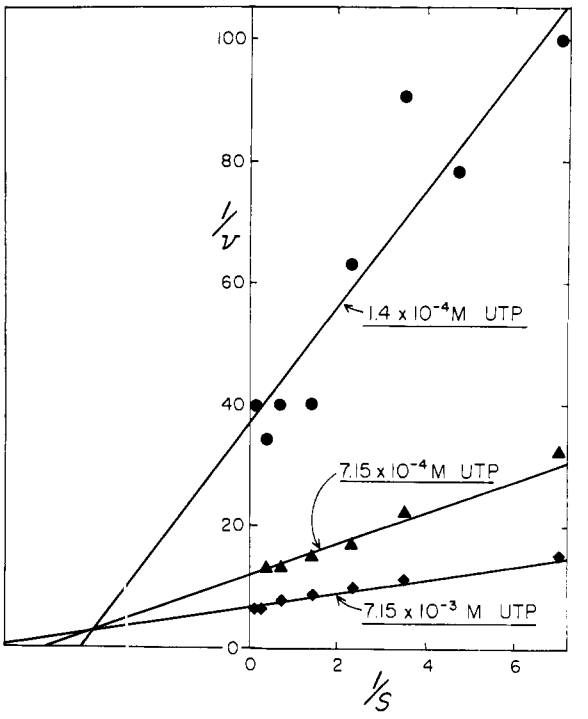

FIGURE 6: Lineweaver–Burk (1934) plot of the response of reaction velocity (arbitrary units) to changes in G-1-P concentration 10^3 M^{-1} at three levels of UTP. (See text for details, assay mixture II.)

low levels of substrates (1.5×10^{-4} M UTP and 7.5×10^{-5} M G-1-P). No significant effect on reaction rate was observed for ATP, AMP, ADP, 3′,5′-cyclic AMP, GTP, GDP, GMP, UDP, UMP, IMP, G-6-P, F-6-P, FDP, DHAP, PEP, R-5-P, glucose, trehalose, citrate, isocitrate, succinate, malate, oxalacetate, glutamate, or inorganic phosphate.

Substrate Concentration in Vivo. The intracellular concentration of G-6-P during development in this organism has been measured, and averages 3.0×10^{-5} M at aggregation and 6.7×10^{-5} M at culmination (see Wright, 1965). Because of the difficulty in accurately measuring the low levels of G-1-P present in extracts of this organism, we have measured the ratio of G-6-P to G-1-P concentrations at these two stages in extracts from large amounts of cells. As Table II shows, this ratio does not change between these stages and has an average value of 5.8. (This is somewhat lower than the value for the equilibrium of phosphoglucomutase obtained in most systems (Najjar, 1962) although a low value has also been recorded in muscle (Larner *et al.*, 1960). The concentrations of G-1-P at aggregation and culmination are calculated to be 0.52 and 1.15×10^{-5} M, respectively.

TABLE II: G-6-P:G-1-P Ratios.[a]

Expt	Stage	G-6-P:G-1-P
I	Agg	5.8
II	Culm	5.0
III	Agg	6.0
IV	Culm	6.4

[a] Determined on concentrated extracts, purified, and assayed spectrophotometrically (see Pannbacker, 1967).

By use of quantitative thin layer chromatographic techniques (see preceding paper) we have measured the concentration of UTP at these two stages of development. The average UTP level at aggregation is 5.0×10^{-5} M and at culmination it is 8.0×10^{-5} M.

Calculation of Theoretical Rates in Vivo. The relative velocity of an enzymatic reaction, ϕ (v/V_{max}) (see Dixon and Webb, 1964), is related to the relative substrate concentration, α ($=S/K_S$), by the relationship $\phi = (\alpha/(1 + \alpha))$. For a two-substrate reaction (in which $\beta = S_2/K_{S_2}$), $\phi = (\alpha/(1 + \alpha))(\beta/(1 + \beta))$. For the case in which $S \ll K_S$, $(\alpha/(1 + \alpha)) = \alpha$; thus, $\phi = \alpha + \beta$.

The amount of enzyme activity in a given volume of packed cells has been measured (extraction performed in the presence of UTP), and at culmination has an average value of 19.3 μmoles/min per ml.

Taking this value as V_{culm}, one can calculate v_{culm} by multiplying this figures by ϕ_{culm}. The relative G-1-P concentration, α_{culm}, is 4.4×10^{-2}; the relative UTP

concentration, β_{culm}, is 1.0×10^{-1}. Thus ϕ_{culm} is 4.4×10^{-3}. This gives a value of 0.085 μmole/min per ml for v_{culm}. The average value for the rate *in vivo* at culmination is 0.075 μmole/min per ml (see preceding paper).

At aggregation V_{agg} = 5.7 μmoles/min per ml, the relative G-1-P concentration, α_{agg}, is 2.0×10^{-2}; the relative UTP concentration, β_{agg}, is 6.2×10^{-2}. Thus ϕ_{agg} is 1.24×10^{-3} and v_{agg} is 0.007 μmole/min per ml. The average value for the rate *in vivo* at aggregation is 0.029 μmole/min per ml. However, if the culmination value for enzyme level is used (that is, assuming no change in enzyme level) a value of 0.024 μmole/min per ml is predicted for v_{agg}.

The above calculations are based on average values determined for a number of different experiments. A certain amount of variability is encountered in all these measurements when different cultures are compared. It was, therefore, felt that all of these measurements should be performed on a single culture. When the concentrations of G-1-P, UTP, and UDPG pyrophosphorylase were compared in such a study at aggregation and early culmination stages, the relative concentration of G-1-P (α_{culm}) was 3.5×10^{-2} at culmination; UTP relative concentration (β_{culm}) was 6.2×10^{-2}. Thus ϕ_{culm} was 2.2×10^{-3}. The observed maximal velocity *in vitro* was 23.6 μmoles/min per ml; thus v_{culm} was calculated to be 0.052 μmole/min per ml. The rate *in vivo* at culmination was 0.031 μmole/min per ml. At aggregation a value of 0.004 μmole/min per ml was calculated for v_{agg} and a rate *in vivo* of 0.015 μmole/min per ml was observed. (A value of 0.014 μmole/min per ml at aggregation is predicted on the basis of enzyme specific activity observed at the culmination stage.)

Discussion

The Significance of Enzyme Level. The observed increase in the rate of UDPG synthesis *in vivo* between aggregation and culmination (~3.5-fold) is not greatly different from the observed increase in enzyme level in extracts (about threefold). One might then conclude that increased enzyme level accounts for the increased reaction rate. However, the change in enzyme level is probably not as great as it appears due to differential instability (Figures 1 and 2). Support for this argument comes from the calculations of the predicted rate *in vivo*. Good agreement between these calculated rates and observed rates *in vivo* is obtained when values for enzyme level at the culmination (stable) stage are used. On the other hand, calculations based on enzyme levels measured at the aggregation (unstable) stage always give low estimates of rate *in vivo*, when compared to those observed rates *in vivo* at this stage. (The agreement between rates *in vitro* and *in vivo* could of course be due to a fortuitous balance between unrecognized factors which exist at the culmination stage and not at the aggregation stage.)

The Significance of Substrate Level. Evaluating the effect of changes in substrate concentration on reaction rates *in vivo* rests on two major assumptions. (1) The kinetic parameters of the enzyme as measured *in vitro* are comparable to its parameters *in vivo*. (2) Substrate levels measured in extracts reflect those levels to which the enzyme is exposed *in vivo*, *i.e.*, there is no significant compartmentalization of enzyme or substrate. With respect to the first assumption our search for effectors uncovered no compounds which seemed to affect reaction rate. The observed agreement between calculated reaction rates and those observed *in vivo* argues that our kinetic parameters are correct.

Compartmentalization could negate the effect of substrate concentration changes in at least two ways. If all the active enzyme and an unchanging amount of substrate were contained in one compartment separated from another in which substrate levels did change, this change would of course not affect reaction rate. We find no evidence that enzyme is compartmentalized, *i.e.*, whether extracted by freezing or gentle homogenization (in 0.01 M buffer or 2.5 M sucrose) the activity is completely soluble when spun at 30,000*g* for 30 min. Furthermore, the specific radioactivities of UDPG and UTP approach the same value during incubation with [^{14}C]uracil (see preceding paper), suggesting that they are not in separate compartments.

A second mechanism by which compartmentalization could negate the effects of changing substrate level is by concentrating all of the substrate into a compartment so small that substrate levels are saturating, *i.e.*, increased substrate level does not affect reaction velocity. To reach this concentration ($10 \times K_m$), UTP and G-1-P would have to be concentrated 100–200 times. The existence of such a small compartment containing all of the UTP and G-1-P in the cell seems unlikely.

It is concluded that increases in substrate levels between aggregation and culmination are sufficient to explain the increased rate of UDPG synthesis observed *in vivo*. This is in agreement with another study of this organism in which increased glutamate oxidation *in vivo* can also be accounted for entirely by increased availability of glutamate (Wright and Bard, 1963; Brühmuller and Wright, 1963).

Considered together, these two studies are very encouraging with respect to the extrapolation of data *in vitro* to explain changes in reaction rate *in vivo*. They also strongly support a major role for substrate availability in the control of processes concerned with differentiation (see Wright, 1966).

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An Assessment of Polynucleotide Inhibition Studies of Aminoacyl-Transfer Ribonucleic Acid Synthetases*

Virginia Zewe Holten† and K. Bruce Jacobson

ABSTRACT: The nature of the interaction between an amino acid and its specific transfer ribonucleic acid (tRNA), as catalyzed by a specific aminoacyl-tRNA synthetase, was investigated using commercially available polynucleotide inhibitors. The basis for such an experimental approach rests upon the hypothesis that this inhibition could involve a specific interaction between the active site of the enzyme and a certain sequence of the polynucleotide corresponding to the recognition site in the tRNA. The results indicate that the data obtained from such experiments are equivocal and must be interpreted with caution. First of all, the degree of polynucleotide inhibition is strongly dependent upon the Mg^{2+} concentration, being much more pronounced under conditions when the cation concentration is limiting. This raises the question as to whether the polynucleotide is inhibiting by virtue of competing with the tRNA for the active site on the enzyme or by Mg^{2+} binding. Secondly, the inhibitory principle in the commercial polynucleotide preparations survived alkaline, acid, and snake venom phosphodiesterase destruction. Thirdly, the criterion of competitive inhibition between the tRNA and the polynucleotide inhibitor as evidence for binding at the active site of the enzyme is questionable, since the metal chelator EDTA also acts competitively with respect to tRNA. Thus, until the metal requirements of each of the components in the system are understood and satisfied, it is not possible to attribute inhibition by polynucleotides to binding at the active site.

A perplexing problem in the area of protein biosynthesis lies in an understanding of the process by which a specific amino acid is attached to a particular tRNA. At present, the nature of this interaction is little understood. One approach to the problem suggested by Hayashi and Miura (1964) involves the use of synthetic oligonucleotides as inhibitors of the aminoacyl-RNA synthetase reaction. A possible mechanism for this inhibition could involve a specific interaction between the active site of the enzyme and a certain sequence of the oligonucleotide corresponding to the recognition site in the tRNA. Thus, those oligonucleotides which function competitively with respect to tRNA would give some indication as to the nucleotide structure at the recognition site of the tRNA molecule.

Recently, several reports which utilize this approach

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