Synthesis of Uridine Diphosphate Glucose Pyrophosphorylase during the Development of *Dictyostelium discoideum*

J. Franke and M. Sussman

From the Department of Biology, Brandeis University, Waltham, Massachusetts 02154

SUMMARY

Uridine diphosphate glucose pyrophosphorylase (EC 2.7.7.9) from *Dictyostelium discoideum* has been purified to apparent physical and immunochemical homogeneity. The molecular weight estimates from gel diffusion and equilibrium sedimentation analyses are 390,000 and 384,000, respectively. The enzyme is polymeric apparently composed of a single monomeric species with a molecular weight of 55,000 as shown by dodecyl sulfate-polyacrylamide gel electrophoresis. The specific activity is 48,000 units per mg. The turnover numbers are 16,500 moles of UDP-glucose and 18,700 moles of glucose-1-P per min per mole of enzyme. The following \( K_m \) values were obtained: glucose-1-P, \( 2.6 \times 10^{-4} \) M; UTP, \( 1.1 \times 10^{-4} \) M; UDP-glucose, \( 1.7 \times 10^{-4} \) M; pyrophosphate, \( 4.4 \times 10^{-4} \) M.

UTP inhibits pyrophosphorylase activity both as a substrate at excess concentration and as a product, the latter inhibition being competitive with UDP-glucose. Pyrophosphate is a potent product inhibitor, not competitive with either glucose-1-P or UTP.

Mixtures of the purified enzyme with crude extracts of cells harvested at different stages of fruiting body construction demonstrated that the assay of enzyme activity employed is an accurate reflection of the concentration of enzyme in the extracts.

A high titer antiserum has been obtained which yields a single band in double diffusion assays with either purified enzyme preparations or crude extracts. Quantitative complement fixation and immune precipitation assays revealed no serological differences between the basal enzyme found in the vegetative cells and that which accumulates during fruiting body construction and demonstrated that the 10-fold increase in the specific activity of the enzyme which occurs during specific stages of fruit construction is correlated with a proportionate increase in a single antigenic component.

The enzyme was also immune precipitated from crude extracts of cells labeled with \[^{35}S\]methionine during the period of enzyme accumulation. Polyacrylamide gel electrophoresis of the enzyme antibody complex solubilized with dodecyl sulfate showed a single labeled peak at the position of the enzyme monomer. Thus at least part if not all of the enzyme that accumulates during fruiting body construction is composed of newly synthesized monomers.

UDP-glucose pyrophosphorylase (EC 2.7.7.9) is a key logistic enzyme in the development of the cellular slime mold *Dictyostelium discoideum*. The fruiting bodies of this species, at specific stages of their construction, accumulate trehalose (1), cellulose (2), glycogen (2), and a mucopolysaccharide containing galactose, N-acetyl galactosamine, and galacturonic acid (2). Together, these carbohydrates eventually comprise about 20% of the dry weight and UDP-glucose serves as a major precursor for their biosynthesis (3-5).

During construction of the fruiting bodies, UDP-glucose pyrophosphorylase activity increases 10-fold. Thus, the cells at the start of this morphogenetic sequence have a specific activity of 40 to 50 units per mg of protein. This basal level is maintained while the heretofore independent cells enter into organized multicellular aggregates and then the enzyme activity rises over a 9- to 10-hour period to a peak of 400 to 450 units per mg. The part of the enzyme activity that is associated with the stalk cells of the mature fruiting body disappears, while the enzyme activity associated with the spores remains. In mutants which display specific morphogenetic aberrations, the pattern of accumulation at the stage at which the development of the mutant diverges from the wild type (4). Experiments involving the use of inhibitors of protein and RNA synthesis have suggested that the pyrophosphorylase is synthesized de novo and is not subject to turnover during the period of its accumulation (4).

The present communication describes the purification of the pyrophosphorylase to apparent physical and immunochemical homogeneity and some of its catalytic, kinetic, and serological properties.

MATERIALS AND METHODS

Organism and Culture Conditions—*D. discoideum*, mutant strain KY-3, was grown in association with *Aerobacter aerogenes* on 2% agar plates containing (per l): 2 g of glucose, 2 g of bacto-

1 A unit is defined as 1 nmole of glucose-1-P produced per min at \( 37^\circ \). (See “Methods” for conditions.)
peptone, 0.2 g of yeast extract, 0.2 g of MgSO₄·7 H₂O, 1.9 g of K₂HPO₄, 1.0 g of KH₂PO₄, and adjusted to pH 6.3 to 6.5. This mutant is morphogenetically deficient and stops development before constructing mature fruiting bodies (6). During the morphogenetic sequence, UDP-glucose pyrophosphorylase activity accumulates in the mutant to the same level as in the wild type (400 to 450 units per mg of protein), but the activity does not disappear as it does in the wild type (4). Thus it is a more convenient source of the enzyme. The pyrophosphorylase has also been purified from the wild type and appears to be catalytically and immunochemically indistinguishable from the KY-3 pyrophosphorylase.

Plates were inoculated (7) in lots of 500 to 1000 and incubated for approximately 80 hours at 22°. By this time the bacterial associate had disappeared and the slime mold amoeba had reached their terminal morphogenetic stage. The cells were harvested in 0.01 M Tris-HCl buffer, pH 7.4, lyophilized, and stored at 5° in vacuum desiccators over silica gel.

Chemicals—Phosphoglcomutase and glucose-6-P dehydrogenase were obtained from Boehringer-Mannheim; Tris, thyroglobulin type I, β amylase from sweet potato, carboxypeptidase A, NADP⁺, NAD⁺, UDP-glucose, glucose-1-P, UTP, and UDP-glucose dehydrogenase (type III) from Sigma; Tricine buffer (N-Tris-hydroxymethylmethylglycine) from Calbiochem; acrylamide gel reagents, BIS (N,N'-methylene bis acrylamide) and TEMED (N,N,N',N'-tetramethyl ethylenediamine) from EM Science; Hypatite-C (hydroxyapatite) from Clarkson Chemical Company; Sephadex G-100 and Sepharose 4B from Pharmacia; cellulose (gelatinized cellulose acetate) from Chemetron, Milan, Italy; crystalline bovine serum albumin from Armour. Where not mentioned all other chemicals were reagent grade and were purchased from Fisher.

Assay Procedures—UDP-glucose pyrophosphorylase catalyzes the reaction: Glucose-1-P + UTP → UDP-glucose + PPᵢ. It was routinely assayed in the direction of glucose-1-P formation by a modification (8) of the method of Munch-Petersen (9). The assay mixture contained in a volume of 1.0 ml: 1 pmole of UTP, 4 pmoles of MgCl₂; 1.6 pmoles of NAD⁺; 85 pmoles of Tricine-NaOH buffer, pH 7.6. Incubation temperature was 37°. The overall reaction is: Glucose-1-P + UTP + UDP-glucose + PPᵢ + NAD⁺ → UDP-glucose + PPᵢ + 2 NADH. One unit of activity is defined as 2.0 nmoles of NAD⁺ reduced per min.

The following buffer solutions were employed and altered the activity of the purified enzyme by no more than 10%; Tricine-KOH, Tris-HCl, Tris-acetate, Tris-maleate. Addition of 0.01 M phosphate, pH 7.6, did not affect the activity.

Purification Procedure—All procedures are carried out at 4°.Lyophilized cells were suspended in water and passed twice through a French pressure cell at 4000 to 6000 p.s.i. The supernatant solution was collected after centrifugation at 16,000 × g for 0 min at 5°. The pH was brought from 6.1 to 7.5 with 0.5 N KOH and 15% streptomycin sulfate solution was added (0.1 v/v). After centrifugation the pellet was washed with 1.5% streptomycin solution, recentrifuged, and the supernatant solutions were combined. This was brought to 46% saturation with saturated ammonium sulfate solution adjusted to pH 7.6 with NH₄OH. The precipitate was removed and the supernatant solution was adjusted to 62% saturation. This precipitate was collected, redissolved in 0.02 M potassium phosphate, pH 7.4, and passed through a Sephadex G-100 column (bed volume, 300 ml; height, 55 cm; flow rate, 30 to 40 ml per hour) with 0.02 M potassium phosphate, pH 7.4. The active fractions which followed closely behind the void volume were pooled and applied to a Hypatite-C column (bed volume, 15 ml, height, 7 cm). The column was successively washed with 0.02 M and 0.07 M potassium phosphate buffer, pH 7.4 (conductivity 2.2 and 7.8 m MHO per cm, respectively), each wash continued until the eluate showed negligible absorbance at 280 nm. To elute the enzyme the column was washed with 0.11 M phosphate, pH 7.4 (12.5 m MHO per cm). The active fraction was concentrated under N₂ in a Diaflo ultrafiltration cell with PM-30 filter at a pressure of 50 p.s.i. UTP was added to a final concentration of 0.01 M to protect the enzyme. The concentrated (20 ml) was dialyzed 2 to 3 hours against 1 liter of 0.075 M Tris-HCl buffer, pH 8.9, and sucrose was added to the dialysate at a final concentration of 10%. The enzyme solution was then applied to a Canalar preparative polyacrylamide gel column (3.5% spacer gel, 4% separator gel) and eluted in 0.375 M Tris-HCl, pH 8.9, at a flow rate of 0.5 to 1 ml per min at 10 mA constant current. The active fractions were pooled, made 1 mM UTP, concentrated to 0.15 mg of enzyme per ml and the concentrate was made 10 mM UTP. Such preparations were stable at 5° for several months.

RESULTS

Criteria of Homogeneity—Table I summarizes the results of a typical preparation. The protein obtained after the last step yielded a single band when stained with Amido black or Coomasie blue after electrophoresis under the following conditions: (a) analytical gel cylinders containing 3, 4, 4.5, and 5% polyacrylamide with Tris-glycine, pH 5.1, Tris-acetate, pH 5.2, and Tris-Cl, pH 8.9, buffers; (b), gel cellophane strips with phosphate or Tricine buffers at several pH values between 6.0 and 8.5.

The protein displayed no detectable heterogeneity when analyzed by the meniscus depletion sedimentation equilibrium method of Yphantis (10). The single component had an apparent molecular weight of 384,000 (calculated by least squares from the points in Fig. 1), assuming a partial specific volume of 0.72.

The preparation gave a single peak of activity with an ap-
**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (units/mg)</th>
<th>Purification Fold</th>
<th>Recovery Step-wise (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin supernatant fraction</td>
<td>970</td>
<td>3700</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>46 to 62% saturated ammonium sulfate fraction</td>
<td>754</td>
<td>420</td>
<td>1,750</td>
<td>76</td>
</tr>
<tr>
<td>Active fraction eluted from Sephadex G-100</td>
<td>517</td>
<td>208</td>
<td>2,500</td>
<td>53</td>
</tr>
<tr>
<td>Active fraction eluted from Hypatite-C</td>
<td>278</td>
<td>31</td>
<td>9,000</td>
<td>29</td>
</tr>
<tr>
<td>Final concentrate after polyacrylamide gel electrophoresis</td>
<td>73</td>
<td>1.5</td>
<td>18,000</td>
<td>26</td>
</tr>
</tbody>
</table>

*a* Protein was estimated by $\text{E}_{280/280}$ nm (21) or by the method of Lowry et al. (22) with bovine serum albumin as standard.

*b* The specific activity of the streptomycin supernatant fraction was approximately equal to that of the original crude extract.

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**Fig. 1.** Analysis of the purified enzyme by the sedimentation equilibrium method of Yphantis (10). The sample (0.15 mg per ml) in 0.34 M Tris-HCl buffer, pH 8.9, with 10 mM UTP was centrifuged in a Spinco model E ultracentrifuge for 39 hours at 9945 rpm. A double sector cell with 12 mm centerpiece was used. The temperature was 7°. Fringe displacement values shown are the means of replicate measurements. Axes: distances in comparator.

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**Fig. 2.** Molecular weight estimate of the final product by gel filtration. A Sepharose 4B column was used (height, 80 cm; volume, 68 ml; flow rate, about 3 ml per hour). One-ml samples were applied and eluted with 0.1 M potassium phosphate buffer, pH 7.2. The column was calibrated with the three proteins shown. These were applied in at least two different concentrations between 1 and 8 mg per ml, individually and in mixtures. The void volume ($V_0$) was determined with Blue Dextran 2000 from Pharmacia; $V_e =$ elution volume. BSA, bovine serum albumin; UDPG, UDP-glucose.

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**Fig. 3.** Quantitative complement fixation analysis of the purified enzyme and partially purified fractions by the method of Wasserman and Levine (23). The complement fixation as percentage of total complement present (\% C'F) is plotted against log$_{10}$ of the units of UDP-glucose pyrophosphorylase activity of the added sample. , streptomycin supernatant fraction, △, 46 to 62% saturated ammonium sulfate fraction, Sephadex G-100 eluate, purified enzyme.

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A high titer antiserum was obtained by repeated injections of the purified enzyme into a rabbit. This serum yielded single nonoverlapping bands in agar double diffusion assays which included the purified enzyme and partially purified fractions from mutant KY-3 as well as preparations from the wild type. Fig. 3 shows the results of quantitative microcomplement fixation assays of the final product and partially purified fractions from mutant KY-3 with 1:4,000 dilution of the antiseraum. The results failed to reveal any appreciable enzymatically inactive component that was active serologically or, conversely, any enzymatically active fraction that was inactive serologically.

**Turnover Number**—A specific activity of about 48,000 units per mg (in the direction of glucose-1-P formation) was obtained with several preparations of purified enzyme. Given the apparent molecular weight of 390,000, the turnover numbers at 37° are estimated at 18,700 moles of glucose-1-P produced per min per mole of enzyme and 16,500 moles of UDP-glucose produced per min per mole of enzyme. At 25° the corresponding values were 13,300 and 8,900, respectively.
Equilibrium Constant—Different concentrations of the substrates in 0.1 M Tricine-NaOH buffer, pH 7.6, and 4 mM MgCl₂ were incubated at 37°C with purified UDP-glucose pyrophosphorylase. At timed intervals aliquots were removed and placed in a boiling water bath for 4 min.

The equilibrium substrate-concentrations were determined by assaying the UDP-glucose with UDP-glucose dehydrogenase and the glucose-1-P with phosphoglucomutase and glucose-6-P-dehydrogenase.

The equilibrium constant \( K_{eq} = \frac{[\text{Glucose-1-P}] [\text{UTP}]}{[\text{UDP-glucose}] [\text{PPi}]} \) was found to be 3 to 4.

pH Optima—Fig. 4 shows that, in the direction of UDP-glucose formation, activity is optimal between pH 7 and 9. In the direction of glucose-1-P formation a relatively sharp peak is seen at pH 7.8. The coupling enzymes present in the assay mixture were shown to be in excess at all pH values tested above.

Rate-Substrate Concentration Curves—Fig. 5 shows the direct and double reciprocal plots of reaction rate versus the concentrations of UDP glucose, pyrophosphate, glucose 1 P, and UTP. All displayed ideal Michaelis-Menten kinetics in the proportional ranges. The \( K_m \) values calculated for these substrates are, respectively, \( 1.7 \times 10^{-4} \); \( 4.4 \times 10^{-4} \); \( 2.6 \times 10^{-4} \); and \( 1.1 \times 10^{-4} \). Among the substrates, only UTP inhibited at high concentration.

Product Inhibition by Pyrophosphate and UTP—Table II summar-

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Fig. 4. The pH dependence of the activity measured in the direction of glucose-1-P (G-1-P) formation, \( \Delta \), and UDP-glucose (UDPG) formation, O. All solutions contained 0.1 M Tricine-NaOH buffer.

Fig. 5. Left, initial velocity of UDP-glucose (UDPG) synthesis with different glucose-1-P (G-1-P) concentrations (upper) and different UTP concentrations (lower). Right, initial velocity of UDP-glucose pyrophosphorolysis with different PPi concentrations (upper) and different UDP-glucose concentrations (lower).
marizes the extent to which PPi and UTP inhibit pyrophosphorylase activity when present as reaction products. The effect of PPi is quite marked; for example 5 mM PPi produced 93% inhibition whereas 3 times that amount had no effect when present as a substrate. The effect of UTP is less marked, about the same as its inhibitory capacity when present as a substrate. In all inhibitory conditions the coupling enzymes were shown to be present in excess.

Fig. 6 summarizes data bearing upon the nature of these inhibitions. The inhibition by UTP is seen to be competitive with UDP-glucose and not competitive with PPi. This interaction had previously been observed with the pyrophosphorylases of human erythrocyte, dog heart, and mung bean (11). The product inhibition of PPi is seen to be not competitive with either substrate. Product inhibition by PPi has not previously been reported for any UDP glucose pyrophosphorylase thus far studied although as a substrate present in excess concentrations it was shown to be a strong inhibitor of the Escherichia coli enzyme (12).

It should also be noted that the enzyme at various stages of purification was found to be protected by UTP (14) and PPi (8).

**Measurements of UDP-glucose Pyrophosphorylase Activity in Crude Extracts**—The developmental kinetics of this enzyme during cellular slime mold fruiting body construction, as described in the introduction, was obtained by assays of crude extracts (4, 8). The data given in Table III indicate that these measurements are valid.

![Graphs](http://www.jbc.org/)

**Fig. 6.** Left, inhibition of UDP-glucose (UDPG) pyrophosphorylase by UTP with UDP-glucose (upper) and PPi (lower) as variable substrates. Right, inhibition of UDP-glucose synthesis by PPi with UTP (upper) and glucose-1-P (G-1-P) (lower) as variable substrates. Note—The last number on abscissas of the figures on the left and top right should be 10 rather than 1.0.
A purified enzyme was preserved in all of the extracts over this period and was the same as when assayed in the purified condition. We therefore conclude that the treatment of crude extracts and conditions of assay used in previous studies (4, 8) provided accurate reflections of UDP-glucose pyrophosphorylase activity at all developmental stages examined.

Immune Precipitation of the Enzyme—Fig. 7 shows the results of immune precipitation assays with a purified enzyme preparation and crude extracts of wild type cells harvested at three stages of fruiting body construction. In the region of antibody excess, no enzyme activity remained in the supernatant after centrifugation of the reaction mixture. (The activity of the complexed enzyme was 20 to 25% of its normal activity.) The data suggest that: (a) the basal enzyme present in the vegetative cells is serologically identical with that which accumulates during morphogenesis; (b) no significant quantity of cross reactive material which is not enzymatically active is present in the crude extracts; and (c) the accumulation of UDP-glucose pyrophosphorylase during fruiting body construction is the same whether measured by enzyme activity or serological activity.

Dissociation of the Enzyme into Subunits—A sample of the purified enzyme was mixed with sodium dodecyl sulfate and mercaptoethanol in 2% final concentrations and incubated in a boiling water bath for 3 to 5 min. Sucrose was added to a final concentration of 10% and the mixture was applied to 6% acrylamide gel equilibrated with 0.1 M sodium phosphate, pH 7.0, and 0.1% sodium dodecyl sulfate (13). After electrophoresis at 10 mA and 50 volts for 3 hours, the gel was fixed and stained with 0.2% Coomassie brilliant blue in 50% methanol, 10% acetic acid and was destained in 7% acetic acid, 0.5% methanol.

The enzyme yielded a single band with a molecular weight of 55,000. The standard proteins used for calibration were: thyroglobulin (170,000); bovine serum albumin, dimeric (136,000) and monomeric (68,000); carboxypeptidase (34,500); rabbit y-globulin H (50,000) and L (23,500) chains (13).

Pulse Labeling of the Enzyme during Fruit Construction—Aliquots of 10^6 cells were deposited on filters (7) and incubated so that they could construct fruiting bodies. After 18 hours the filters were washed with fresh absorbent pads containing [35S]-methionine (1,000 mCi per ml; 20 uCi per ml) dissolved in lower pad solution (Table III) and incubated for 2 hours. Over this period the level of enzyme in the cells rises from about 350 units per mg of cell protein to 450 units per mg (8), i.e. about 25% of the total that accumulates during fruit construction. The cells were then harvested from single filters and lysed with detergent and heated in a boiling water bath with sodium dodecyl sulfate and mercaptoethanol and then subjected to acrylamide gel electrophoresis as described above. The extract from cells harvested at 20.5 hours lost no activity after this treatment. The extract from cells harvested at zero time lost 10% of its activity and the experimental series was corrected for this loss. To obtain extracts, wild type cells were harvested from Whatman filters and treated as described in footnote a, Table III.
of radioactivity. A single peak of radioactivity was obtained whose position coincided with the position of the enzyme subunit. Though a considerable amount of extraneous radioactivity was co-purified with the enzyme, it was distributed at relatively low levels throughout the gel. The results indicate that the accumulation of UDP-glucose pyrophosphorylase is due at least in part to the de novo synthesis of enzyme subunits. Differential rates cannot be estimated since the relative proportions of methionine in the enzyme and in total protein are not known. More extensive experiments with mixtures of labeled amino acids are now underway in order to measure the differential rates of synthesis and turnover, if any.

**DISCUSSION**

UDP glucose pyrophosphorylase has been purified from many sources including mung beans (15), calf liver (16, 17), dog heart and human erythrocytes (11), rat mammary glands (18, 19), and E. coli (12, 20). The *D. discoideum* enzyme has previously been partially purified by Paunovcuk (14) to a specific activity of 1700 units per mg (to be compared with the value of 48,000 units per mg for our own final product). In each case the enzyme turns out to be relatively large. The most precise molecular weight estimates obtained from sedimentation and gel diffusion studies are 480,000 for the crystalline liver enzyme and 440,000 for the erythrocyte enzyme, compared with the value of 390,000 obtained by these methods for the *D. discoideum* enzyme.

Sucrose gradient centrifugation of crude cell extracts has demonstrated the presence of two forms of UDP-glucose pyrophosphorylase differing with respect to sedimentation constants and stabilities (8). The major component comprising at least 90% of the total observed activity is the form that was purified in the present investigation. The minor component, lighter and much less stable, has not been observed in our final product.

The pyrophosphorylase from liver has recently been shown to be polymorphic, probably an octamer (17). Despite the polymorphic structure of the *D. discoideum* enzyme, the rate-substrate concentration curves shown in Fig. 5 all seem to follow the classical Michaelis-Menten kinetics and indicate the absence of ligand site interactions. This is true of all the other pyrophosphorylases as well.

The data shown in Fig. 6 demonstrate that the *D. discoideum* enzyme is subject to product inhibition by UTP and PP. The product inhibition by UTP is competitive with UDP-glucose but noncompetitive with PP. Teuboi, Fukunaga, and Petricciani (11) have previously reported the existence of mutually competitive interactions between UTP and UDP-glucose for the pyrophosphorylases from erythrocyte, dog heart, and mung bean and suggested an ordered bi-bi catalytic mechanism in which the nucleotide is the first substrate to bind and the last product to leave.

The product inhibition by PP, not previously reported for UDP-glucose pyrophosphorylases, is seen to be relatively severe. It is not competitive with either glucose-1-P or UTP thus indicating a separate binding site.

Previous communications have reported that the specific activity of UDP-glucose pyrophosphorylase increases from 45 to 50 to 400 to 450 units per mg of protein during fruiting body construction. The validity of this estimate is confirmed by the experiments summarized in Table III. That this represents a real increase in the quantity of active enzyme is demonstrated by two sets of data: (a) replicate purifications, starting from extracts of vegetative amoebae of *D. discoideum* wild type showing low activity and from fruiting bodies harvested at the peak of activity gave similar percentage yields of purified enzyme and thus confirmed the quantitative differences found in the crude extracts; (b) serological analyses shown in Figs. 3 and 7 demonstrate that the increase in specific activity is correlated with a proportionate increase in the concentration of a single antigenic component all of which can be accounted for as active enzyme. The pulse-labeling data shown in Fig. 8 indicate that this increase in enzyme results in part at least from the de novo synthesis of polypeptide subunits during fruiting. A detailed accounting of these synthetic events is now underway. On the basis of the peak specific activity of 400 to 450 units per mg found in crude cell extracts and the value of 48,000 units per mg for the most purified preparations, it would appear that, at peak, UDP-glucose pyrophosphorylase comprises, at most, 1% of the cell protein.

As mentioned previously, the demand for UDP-glucose during fruiting body construction is considerable and occurs over a relatively brief period. The increased rate of UDP-glucose synthesis might arise at least in part through the 10-fold increase in enzyme specific activity that takes place just prior to and during this period. An alternative has been suggested based on sup-
posed changes in the in vivo, intracellular level of glucose-1-P and UTP (14). The work reported here suggests two alternative mechanisms for the regulation of UDP-glucose pyrophosphorylase activity. One is the possible product inhibition by UDP-glucose competitive with UTP, and the other is the observed product inhibition by PPi.

It should be noted that the analysis of radioactivity of the serological precipitates employed in this study represents a considerable refinement over previous methods (24, 25). Adequate controls demonstrate that when an antigen is precipitated from a labeled crude extract, the amount of unrelated and weakly cross reactive material coprecipitating with the antigen accounts for 50% of the total radioactivity or even more. Treatment of the precipitate with sodium dodecyl sulfate and separation of the monomers of the antigen by acrylamide gel electrophoresis reduce the ambiguity to insignificant levels as shown in Fig. 8. Dr. H. Herschman* has employed the same method to study the synthesis of S-100 protein in human glial cell cultures.

Note Added in Proof—The specific activity value for the purified enzyme has been found to be in error as the result of interference with the protein determination by nondialyzable, extractable components of the preparative acrylamide gel system. Other investigators have reported the same difficulty (26). A better estimate was recently obtained by scanning sodium dodecyl sulfate acrylamide gels of the purified depolymerized enzyme and calibrating with bovine serum albumin. Three different stains (Coomassie blue, fast green, and Amido black) yielded an average value of 100,000 units per mg of protein.

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* H. Herschman, personal communication.

REFERENCES
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