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Uridine Diphosphoglucose Pyrophosphorylase Activity and Differentiation in the Acellular Slime Mold *Physarum polycephalum*

GLENN D. KUEHN

Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003

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The specific activity of uridine 5'-triphosphate:α-D-glucose 1-phosphate uridyltransferase (EC 2.7.7.9) (also called uridine 5'-diphosphate [UDP]-glucose pyrophosphorylase) has been found to increase up to eightfold during spherule formation by the slime mold *Physarum polycephalum*. The enzyme accumulates during the first 8 to 9 h after initiation of spherule formation, declines to basal levels found in vegetative microplasmodia by 15 h, and is undetectable in completed spherules. Specific activities for UDP-glucose pyrophosphorylase in vegetative microplasmodia range from 15 to 30 nmol of UDP-glucose formed per min per mg of protein, whereas accumulated levels during spherule formation can attain a specific activity as high as 125 nmol of UDP-glucose formed per min per mg of protein. The scheduling and extent of accumulation are critically dependent on an early log-phase age of microplasmodia originally induced to form spherules. Spherule induction by 0.2 M or 0.5 M mannitol delays this schedule in a variable and unpredictable manner. Spherule-forming microplasmodia which have accumulated high levels of UDP-glucose pyrophosphorylase spontaneously excrete the enzyme when transferred to salts medium containing 0.2 M or 0.5 M mannitol. The excreted enzyme is subsequently destroyed or inactivated. Studies with preferential inhibitors of macromolecular synthesis indicate that accumulation of UDP-glucose pyrophosphorylase requires concomitant protein synthesis and prior ribonucleic acid synthesis.

Plasmodial differentiation of the slime mold *Physarum polycephalum*, that is, spherule formation, is favored by certain cultural conditions generally involving depletion of utilizable nutrients. Accompanying differentiation are the depletion of glycogen stores, the synthesis of new cell wall components, and the appearance of an extraplasmodial mucopolysaccharide composed almost entirely of polymerized galactosamine (14). Although it is possible to postulate many of the enzymes and precursors that should be involved in this new polysaccharide synthesis, little is known about the level of the requisite enzymes, the actual synthesis intervals for these proteins, or the control of polysaccharide precursor production. Interrelations among some of these factors have been described during the process of aggregation and fruiting-body construction by the cellular slime mold *Dictyostelium discoideum* (6, 7, 10, 20). In particular, studies of factors influencing the expression of the enzyme uridine 5'-triphosphate (UTP):α-D-glucose 1-phosphate uridyltransferase (EC 2.7.7.9), commonly designated as uridine 5'-diphosphate (UDP)-glucose pyrophosphorylase, are providing detailed understanding and fundamentally important methodology toward solution of the general problem of the relationship during development of alterations in enzyme activities and consequent alterations in metabolic fluxes. However, conflicting reports on the levels of developmentally controlled induction of UDP-glucose pyrophosphorylase in *D. discoideum* (1, 6, 16, 22, 24), on methods of assay for the enzyme (6), and on stability of enzyme preparations (6, 15, 23) have frequently detracted from the central problem. Some of the characteristics of expression of this enzyme during spherule formation in the slime mold *P. polycephalum* are described in this paper. The results show that elevated levels of the enzyme accompany differentiation and that in vitro preparations exhibit marked stability, thus offering an attractive system for investigation.

MATERIALS AND METHODS

Organism and culture conditions. The culture of *P. polycephalum* strain CL, used throughout this work, was a gift of D. J. Cooke, University of Leicester, England, and is a homothallic derivative

from a progeny clone of the strain *C50* (21). Shake-cultures of microplasmodia were grown and maintained in 100 ml of semidefined medium plus hematin, as described by Chin and Bernstein (4). Inoculum for spherule formation cultures was prepared by transferring 5 ml of 3-day stock cultures into 100 ml of semidefined medium contained in a 500-ml Erlenmeyer flask, and allowing growth for 24 h to a protein level of about 3 mg/ml of medium. Microplasmodia were allowed to settle in the culture flask and were collected with a large-bore pipette. After centrifugation at 500 × *g* for 30 s, the microplasmodia were washed by again settling through glass-distilled water. After removal of excess wash-water by a second centrifugation, 30 ml of wet-packed plasmodia was transferred to 250 ml of mineral salts medium in a 2-liter Erlenmeyer flask (9). Spherule-forming cultures were subsequently shaken in the dark on a gyratory incubator shaker (New Brunswick Scientific Co., model G25). All procedures above were carried out at 24 C. Under these conditions, spherule formation is completed in at least 35 h. At intervals during spherule formation, designated samples were harvested by centrifugation at 800 × *g* for 30 s, and the pellet was resuspended once in 15 ml of cold deionized water, centrifuged again, and quick-frozen at −70 C. Pellets were stored at −20 C for no more than 1 week prior to enzyme analysis.

In experiments in which spherule formation was induced by the addition of mannitol, similar procedures were used except that the spherule formation medium was supplemented with mannitol as specified (2).

Preparation of extracts. Unfractionated extracts were prepared by suspending the frozen cell pellets in 3 ml of 50 mM *N*-tris(hydroxymethyl)methylglycine (Tricine) (pH 7.5) containing 2 mM ethylenediaminetetraacetic acid, and passing the suspension through a French pressure cell twice at 18,000 lb/in^2. Additional passes through the pressure cell did not increase the concentration of soluble protein. Cell debris was removed by centrifugation at 27,000 × *g* for 20 min. Assays for enzyme activity were performed both immediately after cell breakage and after a minimum dialysis time of 16 h. No significant change in enzyme activity occurred after the latter treatment.

Assays. UDP-glucose pyrophosphorylase catalyzes the following reaction: glucose-1-P + UTP ⇄ UDP-glucose + inorganic pyrophosphate. The enzyme was assayed by a direct isotope method in the direction of UDP-glucose formation from [U-^{14}C]glucose 1-phosphate and UTP. The assay is analogous to that described by Shen and Preiss (18). The enzyme from *P. polycephalum* demonstrated absolute requirements for magnesium ion, UTP, and glucose 1-phosphate for catalytic activity. Reaction mixtures, which contained 15 μmol of Tricine buffer (pH 7.6), 0.8 μmol of $MgCl_2 \cdot 6H_2O$, 0.2 μmol of UTP, and 0.4 μmol of [U-^{14}C]glucose 1-phosphate (specific activity, 4.44 × 10^6 counts per min per μmol), were initiated with addition of specified amounts of enzyme and incubated at 37 C for 5 min. Reactions were terminated by heating the reaction tubes in a boiling-water bath for 30 s. Unreacted [U-^{14}C]glucose 1-phosphate was hydrolyzed to [U-^{14}C]glucose by adding 1.58 units (1 unit is an amount which hydrolyzed 1 μmol of *p*-nitrophenyl phosphate per min at pH 10.4 and 37 C) of *Escherichia coli* alkaline phosphatase (24 μg) in 2 μliters to each tube and incubating the reaction tube at 30 C for 40 min. A 50-μliter sample of each reaction mixture was then applied to diethylaminoethyl-cellulose paper disks (2.5 cm in diameter). Four disks at a time were subsequently washed on a sintered-glass filter with 1 liter of distilled water. These disks were then dried under a heat lamp, placed in a scintillation vial with 5 ml of 0.4% Omnifluor (New England Nuclear) dissolved in a 1:1 (vol/vol) solution of toluene and 2-ethoxyethanol, and counted in a scintillation counter.

The activity of the enzyme was stable under the conditions of the assay for at least 1 h at 37 C and was proportional to total protein concentration up to at least 100 units of enzyme activity per ml in the assay mixture. One unit of UDP-glucose pyrophosphorylase activity is defined as the amount of enzyme that produces 1 nmol of UDP-glucose per min under the conditions above. Corrections have been applied to the calculated rates to account for zero-time controls and background radioactivity.

Protein concentrations were determined by the method of Lowry et al. (13), using crystalline bovine plasma albumin (Pentax) as standard.

RESULTS

Properties of UDP-glucose pyrophosphorylase activity in crude extracts. The formation of UDP-glucose from glucose 1-phosphate is dependent on the simultaneous incubation of an extract of *P. polycephalum*, glucose 1-phosphate, UTP, and $MgCl_2$. Crude extracts derived from microplasmodia harvested at 2-h intervals for 36 h throughout spherule formation failed to demonstrate any activity when 20 mM UTP was substituted by cytidine-, guanosine-, or adenine 5′-triphosphate. The formation of UDP-glucose obeys first-order kinetics until approximately 55% of the added glucose 1-phosphate has been utilized, and is proportional to the amount of protein provided over the range of 0 to 0.3 mg (Fig. 1).

The Michaelis constant (K_m) for UTP at 5 mM glucose 1-phosphate was 2.5 × 10^{-3} M; that for glucose 1-phosphate at 10 mM UTP was 5 × 10^{-4} M (data not shown). These values are quite similar to those reported for the same enzyme from *D. discoideum* (1).

The enzyme from *P. polycephalum* was found to have a broad pH optimum in the range of pH 6.2 to 7.8. It is stable for several days on storage at 5 C in either Tricine buffers or in 0.1 M phosphate (pH 7.0) containing 0.1 M glucose. Dithiothreitol and 2-mercaptoethanol are inhib-

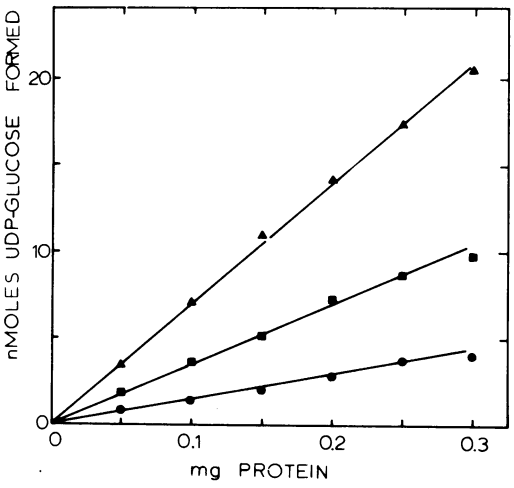

FIG. 1. *Dependence of amount of UDP-glucose formed on crude extract protein in the reaction mixture. Reaction mixtures were prepared as described in Materials and Methods and were incubated at 37 C for 5 min. Extracts were prepared from cells harvested at 0 (●), 8 (■), and 12 (▲) h after initiation of spherule formation.*

itory to the enzyme. These characteristics are the same for enzyme preparations derived from vegetative or spherule-forming microplasmodia.

Changes in UDP-glucose pyrophosphorylase activity during spherule formation. Preliminary experiments demonstrated that a definite culturing protocol was essential to observe increases in UDP-glucose pyrophosphorylase activity after induction of spherule formation. Inoculum from submerged growth cultures had to be in early log-phase and could contain only a minimum of slime. Generally, microplasmodia which had grown longer than 24 h in a single flask did not exhibit increased levels of UDP-glucose pyrophosphorylase activity on transfer to mineral salts starvation medium. Moreover, synthesis of the exomucopolysaccharide slime by microplasmodia growing in the semidefined medium begins as early as 24 h. This can be demonstrated by a simple precipitation test on a small sample of spent, cell-free growth medium. Ethanol, added to 70% concentration, will precipitate as little as 50 μg of polygalactosamine slime in a 1-ml sample. Figure 2 illustrates this dependence of the level of enzyme induction of microplasmodia inoculum age. Microplasmodia, 24 h old, contained UDP-glucose pyrophosphorylase with a specific activity between 15 and 30 units/mg of protein (Fig. 2, circles). On initiating spherule formation by transfer to mineral salts medium, an immediate eightfold increase in specific activity occurred within 6 h. In nine similar experiments, induced levels of pyrophosphorylase have ranged from five- to eightfold. Vegetative microplasmodia older than 24 h failed to accumulate UDP-glucose pyrophosphorylase when induced to form spherules (Fig. 2, squares and triangles).

Figure 2 also shows that the observed rise in UDP-glucose pyrophosphorylase specific activity cannot be ascribed to loss of total cellular protein utilized by starving microplasmodia. The dashed curve shows the variation in total units of enzyme activity, for purposes of comparison, calculated from the data for the 24-h inoculum. Furthermore, during the 6-h interval that UDP-glucose pyrophosphorylase accumulated, the soluble protein which can be isolated from harvested microplasmodia decreases less than 10% (Table 1).

The low levels of enzyme activity observed after 15 h could be due to the presence of an enzyme inhibitor in those samples or to a differential lability of the enzyme. Both of these proposals were found unlikely by a series of mixing experiments (data not shown). Equal amounts of protein from extracts from 8-h induced cells were mixed with extracts from 15-, 25-, and 35-h samples. In all cases, the enzymatic activities of different extracts were strictly additive, with or without prior incubation at 37 C.

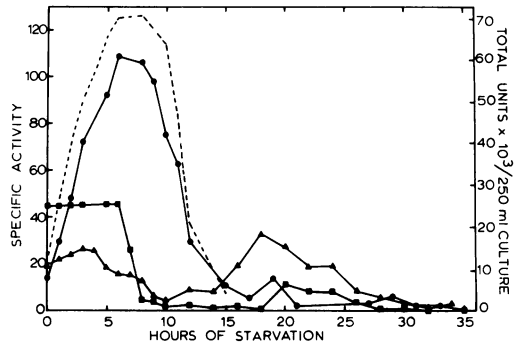

FIG. 2. *Dependence of accumulation of UDP-glucose pyrophosphorylase during spherule formation on the age of the original microplasmodial inoculum. A 5-ml sample of microplasmodia, in 250 ml of mineral salts medium, was harvested at various times after initiation of differentiation and assayed for enzyme activity and protein content. Original growing microplasmodia that had been induced to form spherules were derived from growth cultures which were 24 (●), 36 (■), and 40 (▲) h old. For comparison, the total units of enzyme activity, per 250 ml culture, are shown by the dashed line; these were calculated for the samples indicated by circles.*

TABLE 1. *Soluble protein extracted at pH 7.5 from 3 ml of wet-packed, spherule-forming microplasmodia*[a]

Hours of starvation	Total protein (mg)	Decrease in soluble protein (%)
0	32.7	0
2	32.7	0
3	32.0	2.1
6	30.9	5.5
9	30.0	8.3
13	29.2	10.7
16	26.6	18.7
19	26.8	18.0
22	26.1	20.2
25	23.5	28.1
29	19.4	40.6

[a] At zero h, 94 ml of wet-packed, washed microplasmodia, that had grown 24 h in growth media, was transferred to 250 ml of spherule formation medium in a 2-liter flask. At the times shown, a 10-ml sample of spherule-forming microplasmodia was removed, centrifuged 1 min at 800 × *g*, washed once in cold water, and collected by a second centrifugation. Soluble extracts were prepared and protein analyses were performed as described in Materials and Methods.

Effect of mannitol on UDP-glucose pyrophosphorylase accumulation. Spherule induction by transfer of *Physarum* microplasmodia to a defined salts medium containing 0.5 M mannitol reportedly accelerates differentiation (2). Thus it was of interest to determine the effect of this polyol on UDP-glucose pyrophosphorylase expression during the early stages of spherule formation. At all mannitol concentrations tested above 0.20 M, microplasmodia abruptly lost 80 to 90% of their UDP-glucose pyrophosphorylase activity on transfer from growth conditions to starvation medium containing mannitol. In fact, harvesting and sampling methods were not sufficiently rapid to detect intermediate enzyme activities during the course of decline. UDP-glucose pyrophosphorylase induction also occurred in these depleted cultures although at a delayed and variable interval despite standardization of culturing procedures. Figure 3 is typical of numerous experiments. Generally, the period of accumulation extended over 20 h and variations in activity were observed as late as 60 h after initiation of spherule formation. The maximum level of induced activity never exceeded four times that of the original microplasmodial inoculum and ranged in occurrence as early as 15 h to as late as 55 h from transfer to starvation medium. Chet and Rusch (2) have shown that [^{14}C]mannitol is not incorporated into cellular carbohydrate or protein constituents by spherule-forming *P. polycephalum*, although microplasmodia are apparently permeable to this polyol. Thus, the possibility of mannitol catabolism with concomitant delay of UDP-glucose pyrophosphorylase induction did not appear to explain the results presented in Fig. 3.

In addition, microplasmodia which had been induced to form spherules in the absence of mannitol for 9.75 h similarly lost their maximally-induced levels of UDP-glucose pyrophosphorylase on transfer to a salts medium plus 0.2 M or 0.5 M mannitol. This is shown in Fig. 4 (circles) where the total enzyme units are plotted versus time.

In an attempt to account for this rapid enzyme loss, the spherule formation medium from which cell samples had been harvested was assayed for UDP-glucose pyrophosphorylase activity. New enzyme activity appeared in the mannitol-containing medium immediately after transfer from the mannitol-free medium (Fig. 4, triangles). Thirty minutes after transfer, less than 5% of the activity could be detected in the microplasmodia and only 64% was found in the external medium. Apparent destruction or inactivation of the enzyme occurred rapidly after excretion. The nature of this process has not been investigated, although excretion of a single, potent protease accompanies spherule formation (unpublished observation) and may mediate this inactivation.

Effect of cycloheximide and actinomycin D on UDP-glucose pyrophosphorylase accumulation. Cycloheximide has previously been

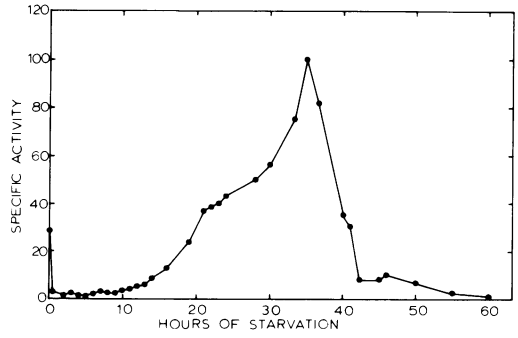

FIG. 3. *Effect of mannitol on the accumulation of UDP-glucose pyrophosphorylase activity. Growing microplasmodia were washed and transferred to 250 ml of spherule formation medium containing 0.5 M mannitol at zero time. At various times indicated, a 5-ml sample was harvested and the microplasmodia were asssyed for enzyme activity and protein content.*

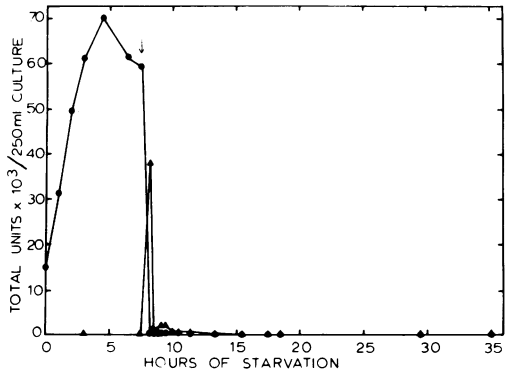

FIG. 4. *Mannitol-induced excretion of UDP-glucose pyrophosphorylase from spherule-forming microplasmodia. Growing microplasmodia were washed and transferred to 250 ml of spherule formation medium, minus mannitol, at zero time. At various times, 5-ml samples were harvested and the microplasmodia were asssyed for enzyme activity and protein content (●). After 9.75 h, the microplasmodia in the main culture were harvested and transferred to spherule formation medium containing 0.5 M mannitol (arrow). At times shown, microplasmodia (●) and accompanying extracellular medium (▲) were sampled and analyzed for enzyme activity.*

shown to inhibit protein synthesis preferentially in *P. polycephalum* plasmodia without significantly affecting ribonucleic acid (RNA) synthesis (5, 17). The affect of inhibiting protein synthesis by cycloheximide on UDP-glucose pyrophosphorylase activity during the early stages of spherule formation is shown in Fig. 5. Clearly the accumulation of enzyme activity requires concomitant protein synthesis. The addition of cycloheximide (10 μg/ml) to cultures any time up to 9 h after spherule induction caused an abrupt decline in UDP-glucose pyrophosphorylase activity. The approximate half-lives of decay in enzyme activity after administration of inhibitor at 2, 4, and 9 h, were 5.6 h, 2.2 h, and 2.0 h, respectively.

Actinomycin D has been shown to inhibit RNA synthesis by more than 80% during growth and spherule formation of *P. polycephalum* and generally blocks the morphogenetic process (2). It also inhibits the incorporation of ^{3}H-labeled protein hydrolysate during spherule formation, but not during growth, indicating that new RNA synthesis is essential for the translation process during spherule formation (3). Thus, the relation of RNA synthesis to accumulation of UDP-glucose pyrophosphorylase was determined by transferring spherule-forming microplasmodia at various times to medium containing actinomycin D (300 μg/ml) and measuring the subsequent change in specific activity. The results (Fig. 6) show that the increase in specific activity of UDP-glucose pyrophosphorylase proceeds normally for about 3 h after administration of inhibitor, and then ceases abruptly. Moreover, if exposure to actinomycin D is begun before the sixth hour after initiating spherule formation, UDP-glucose pyrophosphorylase activity is apparently stable after cessation of accumulation (Fig. 6, triangles and squares). Administration of inhibitor after 6 h results in a rapid decline in specific activity 3 h later (Fig. 6, crosses).

DISCUSSION

Spherule development of *P. polycephalum* occurs in about 35 h. During the first 15 h of accompanying starvation, glycogen content decreases about 70% (8). The metabolic fate of this carbon source is largely unknown, although it seems likely that various specific carbohydrates which characterize morphogenesis of *P. polycephalum* could be synthesized from glucose 1-phosphate derived from storage glycogen (14).

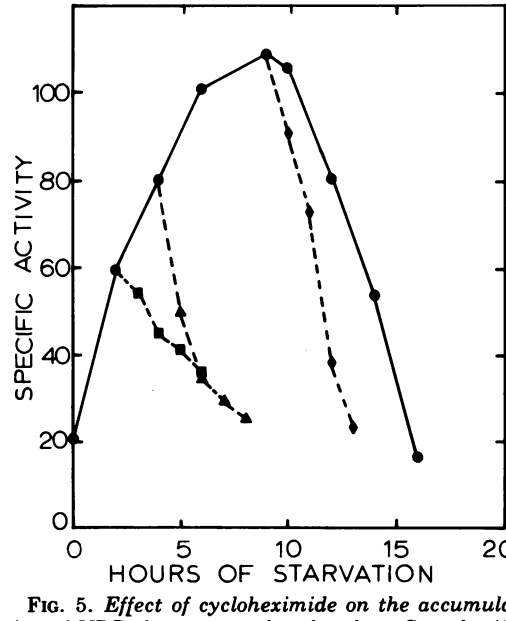

FIG. 5. *Effect of cycloheximide on the accumulation of UDP-glucose pyrophosphorylase. Samples (50 ml) of microplasmodia differentiating in 250 ml of salts medium (●) were harvested and transferred at 2 (■), 4 (▲), and 9 (◆) h to 50 ml of fresh salts medium containing cycloheximide (10 μg/ml). Samples (5 ml) of microplasmodia, from both control and experimental cultures, were harvested for protein and enzyme analysis at the succeeding times indicated.*

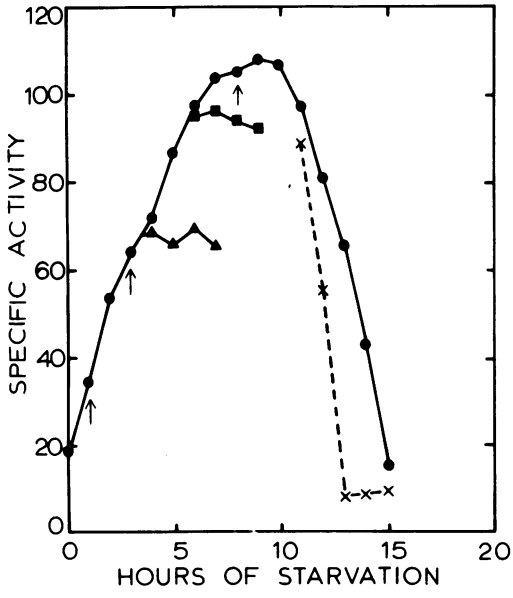

FIG. 6. *Effect of actinomycin D on the accumulation of UDP-glucose pyrophosphorylase. Samples (50 ml) of microplasmodia differentiating in 250 ml of salts medium (●) were harvested and transferred 1, 3, and 8 h after initiation of spherule formation (see arrows), to 50 ml of fresh salts medium containing actinomycin D (300 μg/ml). At 3 h after transfer to salts medium containing actinomycin D, 5-ml samples of microplasmodia, from both control (●) and experimental cultures (▲, ■, and ×), were harvested for protein and enzyme analysis at the times indicated.*

The above results demonstrate that UDP-glucose pyrophosphorylase accumulates within the first 8 to 9 h of spherule formation to a specific activity more than eight times that found in growing plasmodia. The specific activities of several other enzymes of carbohydrate and amino acid metabolism have also been found to change during spherule formation (11, 12), including isocitrate dehydrogenase, glucose 6-phosphate dehydrogenase, histidase, β-glucosidase, acid phosphatase, *N*-acetylglucosamine-4-epimerase, glutamate dehydrogenase, and phosphodiesterase. Only the latter two demonstrated levels of accumulation comparable to that reported here for UDP-glucose pyrophosphorylase, and these increases occurred gradually throughout a 24-h interval. In contrast, accumulation of UDP-glucose pyrophosphorylase begins almost immediately after differentiation is initiated in growing microplasmodia and lasts only 8 to 9 h. The dependence of this observation on inoculum age should be emphasized (Fig. 2). Recently, *N*-acetylglucosamine-4-epimerase activity was reported to change little throughout spherule formation in *P. polycephalum* (11). However, as our results show, both the sampling schedule and inoculum age employed in these experiments would probably preclude the possibility of detecting induction of new epimerase activity.

Transfer of growing and spherule-forming microplasmodia to salts medium containing mannitol causes aberrations in the schedule of accumulation of UDP-glucose pyrophosphorylase activity. Immediate loss of existing activity is followed by later unpredictable accumulation. Similar variable behavior in mannitol-medium has been observed for expression of an intraplasmodial and an extraplasmodial *N*-acetylglucosaminidase and β-glucosidase activities in *P. polycephalum* (unpublished observation). These observations, at a molecular level, agree with findings of others (11) and demonstrate that in studies of this type the use of polyols for spherule induction of *P. polycephalum* is not a useful technique.

The dilemma inherent in interpretation of developmental enzymatic studies that employ crude cell extracts and inhibitors of macromolecular synthesis is apparent. However, assuming specificity for the sites of inhibition by cycloheximide and actinomycin D, the results of this report agree with the notion that accumulation and decline in UDP-glucose pyrophosphorylase activity are subject to both transcriptional and translational controls. Accumulation of the enzyme activity requires concomitant protein synthesis (Fig. 5). RNA synthesis essential for continued rise in activity precedes actual expression of the enzyme activity by at least 3 h (Fig. 6). Whether or not these controls act directly at the level of synthesis of UDP-glucose pyrophosphorylase molecules cannot be determined.

It may be significant that the interval of rise and decline for UDP-glucose pyrophosphorylase activity (Fig. 2) coincides with the period in which glycogen in spherule-forming microplasmodia of *P. polycephalum* decreases by 70% (8). However, these events are inconsistent with the timing and extent of accumulation of the mucopolysaccharide slime itself outside developing spherules. Maximum extracellular slime synthesis occurs approximately 18 h after the initiation of starvation (8) at a time when assayable levels of UDP-glucose pyrophosphorylase are lowest. Thus, the flow of carbohydrate in spherule-forming *P. polycephalum*, from the time of its disappearance as glycogen to its alleged reappearance in new cell wall

components and extracellular slime, appears to be discontinuous. Moreover, extensive attempts in our laboratory have failed to demonstrate anticipated activities for either UDP-galactose:polysaccharide galactosyl transferase (19) or UDP-*N*-acetyl-galactosamine:polysaccharide *N*-acetyl-galactosamine transferase enzymes in microplasmodia derived from all stages of spherule formation. These observations suggest the existence of an intermediate pool of carbon precursors to which glycogen carbohydrate is converted prior to actual synthesis of extracellular polysaccharide.

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