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Developmental Regulation of α-Mannosidase in *Dictyostelium discoideum*

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The specific activity of α-mannosidase (EC 3.2.1.24) has been found to increase more than a thousandfold during development of the cellular slime mold, *Dictyostelium discoideum*. The enzyme accumulates in both spore and stalk cells. Studies with preferential inhibitors of macromolecular synthesis indicate that accumulation of α-mannosidase requires concomitant protein synthesis and prior ribonucleic acid synthesis. Control of the period of synthesis by the overall developmental program is demonstrated in two temporally deranged morphological mutants. α-Mannosidase is found in lysosomes of *D. discoideum* in association with other acid hydrolases which may be involved in metabolism of extracellular polysaccharide.

When development is initiated in *Dictyostelium discoideum*, the amoebae aggregate and form multicellular migrating pseudoplasmodia which proceed through a series of morphological stages culminating in the formation of sorocarps. Each sorocarp consists of up to 5×10^4 spores supported on a tapering cellular stalk. The integration of previously identical free-living cells of *D. discoideum* into a single differentiating organism may be mediated by the extracellular slime which surrounds the organism. This polysaccharide is excreted by the cells and remains stationary with respect to the surface over which the pseudoplasmodium is traveling (13, 15). The tip cells control the direction of migration by laying down slime on which posterior cells must travel. Thus, the excreted polysaccharide plays a decisive role in the integration of cellular movements and may also be involved in the regulation of morphogenesis.

An extracellular polysaccharide has recently been isolated from amoebae and migrating pseudoplasmodia (4; Loomis, *unpublished data*) and shown to consist of *N*-acetylglucosamine (70%), fucose (24%), and mannose (6%). Further understanding of the role of this polysaccharide might result from study of the enzymes involved in slime metabolism in *D. discoideum*.

Previous work in this laboratory has shown that the specific activity of β-*N*-acetylglucosaminidase (EC 3.2.1.30) increases dramatically during the first 8 hr of development (7). The involvement of this enzyme in slime metabolism is suggested by the discovery that mutant strains which fail to accumulate acetylglucosaminidase also fail to aggregate (7, 9). This provided encouragement for an analysis of the developmental control of mannosidases and fucosidases. Little activity was found at any developmental stage toward nitrophenyl derivatives of β-D-mannoside, α-L-fucoside, or β-D-fucoside. However, considerable activity toward nitrophenyl α-D-mannoside was found in developing cells. An analysis of the developmental kinetics of α-mannosidase showed that the specific activity increased by more than a thousandfold from aggregation to sorocarp formation. The results presented here indicate that this enzyme is an integral part of normal development which may be involved in metabolism of the extracellular polysaccharide.

MATERIALS AND METHODS

Chemicals. *p*-Nitrophenyl α-D-mannopyranoside was purchased from Pierce Chemical Co., Rockford, Ill. Cycloheximide was the product of the Upjohn Co., Kalamazoo, Mich. Actinomycin D was purchased from Merck and Company, Inc., Rahway, N.J.

Organisms. *D. discoideum* NC-4 (haploid) was isolated and described by Raper (12). All mutant strains employed in this study were derived from this strain. Strains Agg 206, KY-3, KY-19, FR-17 were isolated in the laboratory of Maurice Sussman and have been described (8). Strain DA-2 was isolated in this laboratory by Daphne Allister after treatment of strain NC-4 with the mutagen *N'*-methyl-*N'*-nitro-nitrosoguanidine. The mutant strain fails to aggregate. Strain GN-3 was isolated in this laboratory and has been described (9).

D. discoideum was grown on SM agar plates in association with *Aerobacter aerogenes* (18). Development was initiated by collecting the amoebae from growth plates, washing them free of bacteria by differential centrifugation, and depositing 10^8 cells

on single-membrane supports (AABP; Millipore Corp., Bedford, Mass.) resting on pads saturated with 2 ml of a solution containing NaCl (9 mg/ml), streptomycin sulfate (0.5 mg/ml), and 10^{-3} M potassium phosphate buffer (*p*H 6.5).

α-Mannosidase assay. At intervals after the initiation of development, the cells were collected off a single-membrane support in 3 ml of distilled water and frozen until needed for assays. The samples were then thawed and ultrasonically disrupted with a Bronson sonifier at a tip energy of 40 w for 30 sec. α-Mannosidase was assayed within an hour. This procedure resulted in maximal measurable activity throughout the developmental sequence.

Samples of sonically treated extracts were incubated with 5×10^{-3} M *p*-nitrophenyl α-mannoside (Pierce Chemical Co., Rockford, Ill.) in 5×10^{-2} M acetate buffer (*p*H 5). After incubation for 30 min at 35 C, the reaction was stopped by addition of an equal volume of 1 M Na_2CO_3. Nitrophenol formation was estimated by measuring the absorbance at 420 nm on a Zeiss spectrophotometer with the appropriate blanks. The assay is linear with extract containing 10 to 100 μg of protein and is linear with time for more than 24 hr. One unit of activity is defined as that amount which will liberate 1 nmole of nitrophenol per min under the above conditions. Specific activity is expressed as units per milligram of protein. Protein was estimated by the method of Lowry et al (11) by using crystalline bovine serum albumin as a standard.

Acrylamide gel electrophoresis. Samples in 15% sucrose were applied to 6-cm 5% acrylamide gels polymerized by 0.025% *N,N,N',N'*-tetramethyl ethylenediamine and 0.07% ammonium persulfate in 0.05 M sodium phosphate buffer (*p*H 7.2). The tray buffer was 0.10 M sodium phosphate (*p*H 7.2; reference 2).

The sample was "focused" (2) at 0.5 ma per gel for 15 min, and then electrophoresed at 5 ma per gel for about 115 min at 4 C. The gels were extruded and sliced into 2-mm fractions; each fraction was assayed in 0.2 M acetate (*p*H 5.0) for α-mannosidase activity.

Chromatography. The products of the α-mannosidase assay reaction were chromatographed with authentic compounds on Eastman 6065 cellulose thin layers in a solvent of *t*-butyl alcohol-methyl ethyl ketone-formic acid-water (8:6:3:3; reference 3). Nitrophenyl derivatives could be located under ultraviolet light by using the fluorescent indicator in the thin layers. Reducing sugars were located with a silver nitrate stain (21).

RESULTS

Although little α-mannosidase activity could be measured in growing vegetative cells, both spores and stalk cells contained considerable activity (Table 1). The enzyme was found to have two *p*H optima, one at *p*H 3.5 and one at *p*H 5 (Fig. 1). Since several other acid hydrolases of *D. discoideum*, including *N*-acetylglucosaminidase, acid phosphatase (EC 3.1.3.2), and β-glucosidase (EC 3.2.1.30), have been shown to be localized in lysosomes (Weiner and Ashworth, *in press*; Coston, *unpublished data*), it seemed likely that α-mannosidase would be similarly localized. To determine the compartmentalization of this enzyme, developing cells were broken in isoosmotic solution (0.25 M sucrose), and the lysosomes were pelleted by centrifugation at 27,000 × *g* for 10 min. Assays of the supernatant fraction and resuspended pellet showed that 83% of the α-mannosidase activity cosedimented with the lysosomes. When the pelleted lyosomes were subsequently lysed by suspension in distilled water, 73% of the associated α-mannosidase activity was released into the soluble fraction, confirming that the majority of α-mannosidase is confined in lysosomes.

TABLE 1. *Specific activity of α-mannosidase in various cell types*

Cell type	α-Mannosidase units/mg of protein
Vegetative amoebae	0.028 ± 0.005
Mature fruiting body	36 ± 4
Spore cells	32 ± 4
Stalk cells	29 ± 3

To determine the number of protein species with α-mannosidase activity, the extracts of developing cells were analyzed by polyacrylamide gel electrophoresis. Approximately 2×10^9 cells were collected from membranes supports 18 hr after the initiation of development into 3 ml of distilled water. The cells were broken on a Bronson sonifier, and the extract was centrifuged at 48,000 × *g* for 1 hr. The supernatant fraction was then dialyzed, layered on a 5% polyacrylamide gel, and electrophoresed. The gel was then fractionated and assayed for α-mannosidase (Fig. 2). It is clear that *D. discoideum* contains only a single electrophoretic form of α-mannosidase.

The enzyme was purified 20-fold from sonically treated extracts of developing pseudoplasmodia by gel filtration. α-Mannosidase activity eluted from a column of Sephadex G200 as a single symmetrical peak shortly after the excluded proteins. When chromatographed on Sephadex G100, the enzyme eluted with the excluded volume.

The Michaelis constant (K_m) for *p*-nitrophenyl α-D-mannoside of the enzyme in crude extracts or after 20-fold purification was found to be 10^{-3} M under standard assay conditions (Fig. 3). When 1 μmole of substrate was hydrolyzed under standard assay conditions by partially purified enzyme, the only reducing compound co-chromatographed with authentic mannose.

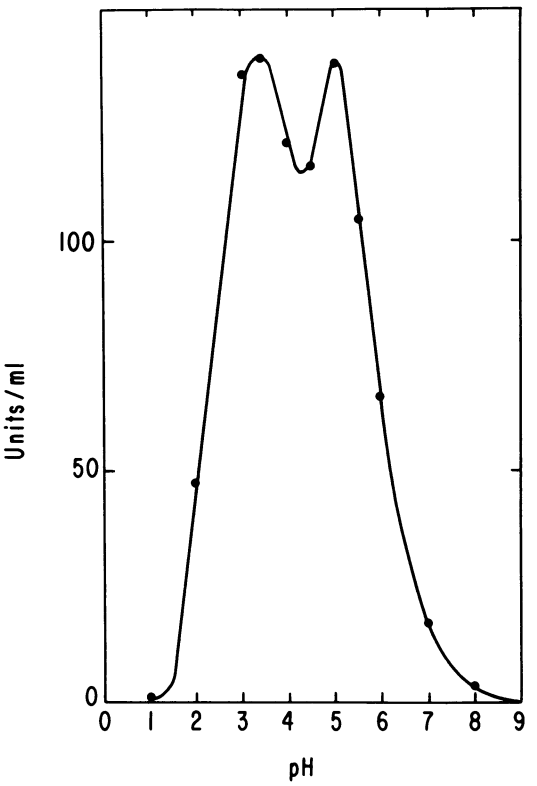

FIG. 1. *Optimal pH levels for α-mannosidase. Extracts of cells which had developed for 20 hr were incubated under standard assay conditions except that the pH was adjusted with appropriate buffers: pH 1 to 4, glycine-HCl at 5 × 10⁻³ M; pH 4.5 to 6, acetate at 5 × 10⁻³ M; pH 7 to 8, potassium phosphate at 5 × 10⁻³ M.*

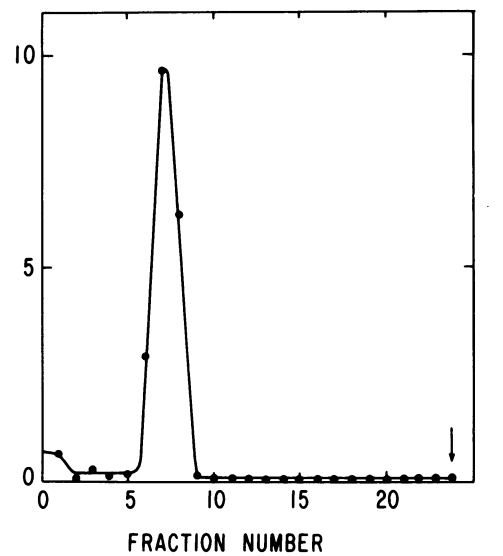

FIG. 2. *Acrylamide gel electrophoresis. Dialyzed extract of culminating cells was layered on a 6-cm gel and electrophoresis was for 2 hr as described. Fractions (2 mm) were then collected and assayed for 130 min. The enzyme migrates toward the anode at the right. The arrow indicates the position of the front as shown by bromphenol blue added to a parallel gel.*

Kinetics of accumulation of α-mannosidase. Development of *D. discoideum* occurs in about 26 hr. During this process the specific activities of several developmentally controlled enzymes increase during discrete periods of about 10 hr (1, 7, 8, 14). α-Mannosidase, however, was found to accumulate throughout the entire developmental process (Fig. 4). The increase in specific activity only ceased when the cells had completed cytodifferentiation into spores and stalk cells at which stage all metabolic activities are greatly reduced (5). It is clear that, although the products of some genes accumulate only at certain stages of development, the α-mannosidase gene product accumulates at an essentially constant rate during aggregation, pseudoplasmodium formation, and culmination.

The accumulation of α-mannosidase requires concomitant protein synthesis since the addition of cycloheximide at the time of initiation of development or 9 hr later blocked all subsequent

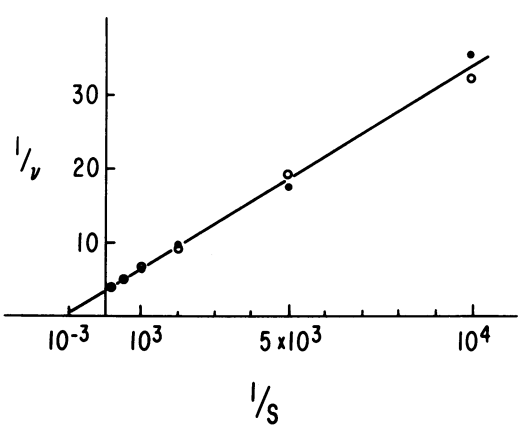

FIG. 3. *Determination of the Michaelis constant (K_m) for nitrophenyl α-mannoside. Crude extracts of cells which had developed for 18 hr at 22 C were incubated under standard assay conditions with various amounts of nitrophenyl α-mannoside and the rate of substrate hydrolysis was determined (●). Similar determinations were performed by using enzyme purified by chromatography on a Sephadex G-200 column (○). The data are plotted as suggested by Lineweaver and Burk (6). S refers to the final concentration of nitrophenyl α-mannoside in the assay. Velocity (v) is given as units/ml and has been multiplied by 25 for the experiment with purified enzyme for convenience in presentation.*

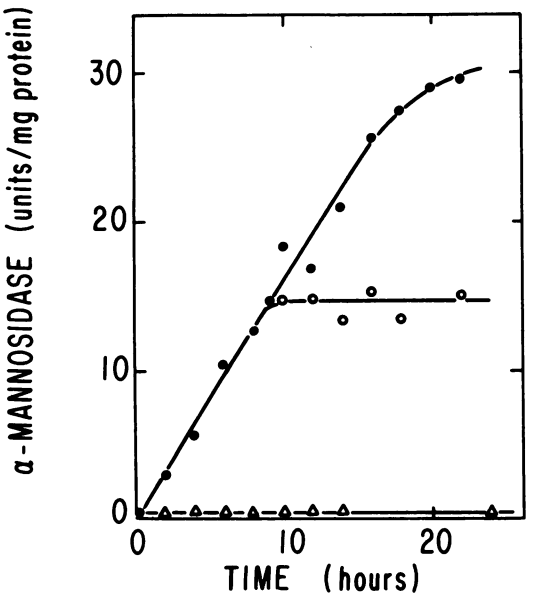

FIG. 4. *Developmental kinetics of α-mannosidase Cells of D. discoideum were collected from membrane supports at various times after the initiation of development. The specific activity of α-mannosidase was determined in control cells (●) and in cells treated with cycloheximide (400 μg/ml) at 0 (△) or 9 hr (○) after initiation of development.*

increase in specific activity (Fig. 4). Cycloheximide has been previously shown to preferentially inhibit protein synthesis in *D. discoideum* (17). Preformed enzyme appears to be stable in the absence of protein synthesis (Fig. 4).

RNA synthetic period for α-mannosidase. The accumulation of several enzymes in *D. discoideum* has been shown to require ribonucleic acid (RNA) synthesis after the initiation of development (1, 7, 8, 14). The periods during which RNA synthesis must occur for the individual enzymes varies from 2 to 14 hr before the increase in the respective specific activities. The relation of RNA synthesis to accumulation of α-mannosidase was determined by adding actinomycin D at various times and measuring the subsequent increase in specific activity. Actinomycin D has been previously shown to inhibit RNA synthesis at least 95% within 30 min without significantly affecting protein synthesis for more than 4 hr (19). The results (Fig. 5) show that the increase in specific activity of α-mannosidase proceeds normally for about 3 hr after the inhibition of RNA synthesis and then abruptly ceases. It appears that RNA for α-mannosidase is synthesized several hours before translation into active enzyme.

Relation to morphogenesis. Control mechanisms operative in the initiation of some biochemical differentiations in *D. discoideum* have been shown to be dependent on conditions which result from multicellular morphogenesis (8, 10). The synthesis of several other developmentally controlled enzymes is independent of intercellular interactions (9, 10). To determine the requirements for continued accumulation of α-mannosidase, pseudoplasmodia were dissociated to single cells 14 hr after the initiation of development. Although the cells required about 4 hr to reaggregate and reform pseudoplasmodia, α-mannosidase continued to accumulate without interruption (Fig. 6). It is clear that the synthesis of this enzyme is independent of multicellular topography.

Developmental kinetics in morphological mutants. Since accumulation of α-mannosidase occurs almost immediately after development is initiated in the individual growing amoebae and does not require subsequent multicellularity, we would not expect aggregation and morphogenesis to be required for the increase in specific activity of α-mannosidase. This expectation was tested in a series of morphological mutants in which

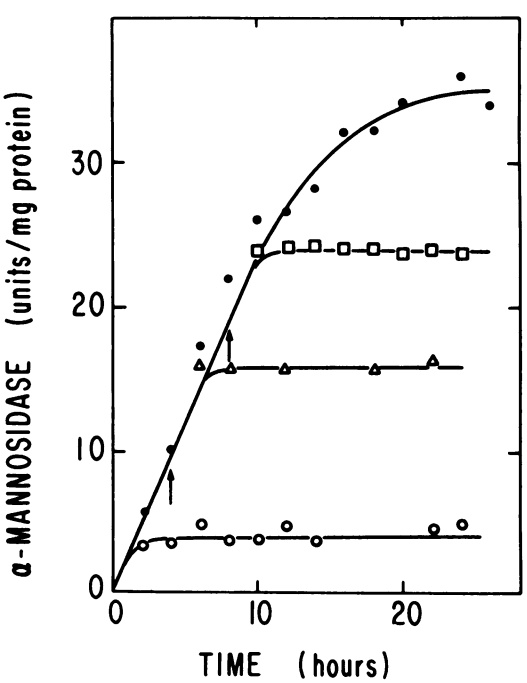

FIG. 5. *Effect of actinomycin D on the accumulation of α-mannosidase. Cells developing on membrane supports were shifted to fresh pads saturated with 125 μg of actinomycin D per ml at 0 (○), 4 (△), and 8 (□) hr after initiation of development. The arrows indicate the time of addition of the drug. Control cells (●) were not shifted.*

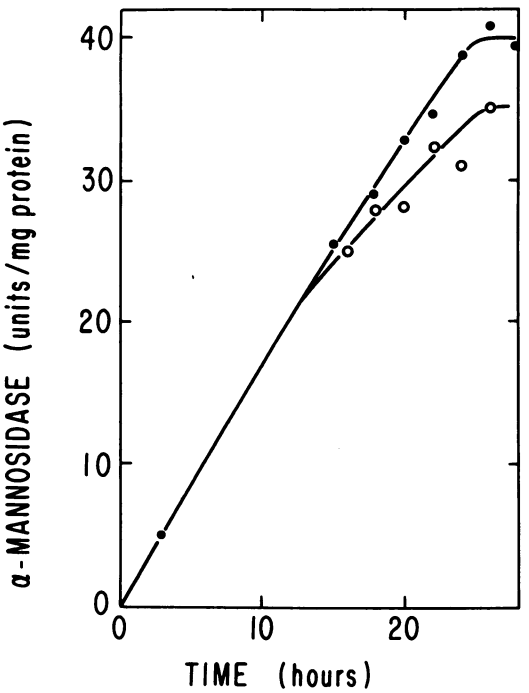

FIG. 6. *Effect of multicellular association on the accumulation of α-mannosidase. Pseudoplasmodia which had developed for 14 hr at 22 C were dissociated to single cells and small groups of cells by trituration. The dissociated cells were redeposited on fresh membrane supports and incubated at 22 C. Samples were taken for assay of α-mannosidase activity and protein content. Control cells (●); dissociated cells (○).*

various steps in cytodifferentiation are genetically impaired (Table 2). α-Mannosidase increased to approximately the normal specific activity in strains blocked in fruiting body formation (KY-3) or stalk formation (KY-19) and accumulated to more than half the normal amount in two strains which fail to aggregate (206 and DA-2). Thus, it seems that none of the steps in morphogenesis is required for the accumulation of α-mannosidase, and it is possible that the enzyme is not part of the overall developmental pattern. However, α-mannosidase could be shown to be included in the sequence of biochemical differentiations in two temporally deranged mutant strains. Strain FR-17 was isolated several years ago by Sonneborn et al. (16) and shown to proceed through all the steps of biochemical differentiation in less than two-thirds the time required by wild-type cells. Strain GN-3 was recently isolated (9) and shown to develop at less than a third the normal rate. The developmental kinetics of α-mannosidase in these mutant strains were determined (Fig. 7). It is apparent that, like other developmentally controlled enzymes, α-mannosidase accumulates precociously in strains FR-17 and belatedly in strain

TABLE 2. *α-Mannosidase in morphological mutants*

Strain	Phenotype	Maximal specific activity (α-mannosidase units/mg of protein)
NC-4	Wild type	38
Agg 206	Aggregateless	20
DA-2	Aggregateless	26
KY-3	Culmination defective	42
KY-19	Stalkless	38
FR-17	Rapid development	30
GN-3	Slow development	52

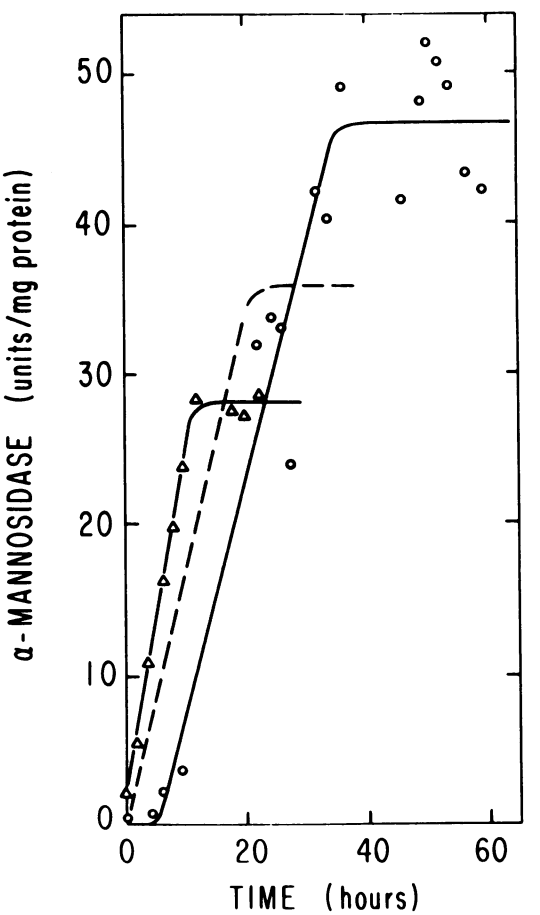

FIG. 7. *α-Mannosidase in temporally deranged mutants of D. discoideum. The specific activity of α-mannosidase was determined at various times after the initiation of development in strain FR-17 (△) and strain GN-3 (○). The accumulation of α-mannosidase in wild-type cells is indicated by the dotted line.*

GN-3. It was concluded that the accumulation of α-mannosidase is subject to the integrating temporal mechanisms which control development in *D. discoideum*.

DISCUSSION

The description of a series of developmentally controlled enzymes provides markers for the biochemical analysis of development of *D. discoideum*. Changes in the various enzymes after genetic, chemical, or mechanical perturbation of normal morphogenesis gives evidence on the causal relationships operative during the various stages of cytodifferentiation (9, 10, 20). The kinetics of accumulation of α-mannosidase can be compared with those of other enzymes presently known to be controlled in *D. discoideum* (Fig. 8). Although other enzymes rise from basal to peak activities during approximately 10 hr, α-mannosidase accumulates for 20 hr. Since the turnover numbers for these enzymes have not been determined, the actual rate of accumulation as expressed in molecules per minute cannot be derived from these data. Thus, it is quite possible that, on a molecular basis, α-mannosidase is synthesized as rapidly as other developmentally controlled enzymes but for twice as long. Moreover, it must be emphasized that the peak specific activities determined in vitro vary up to 10-fold for the individual enzymes.

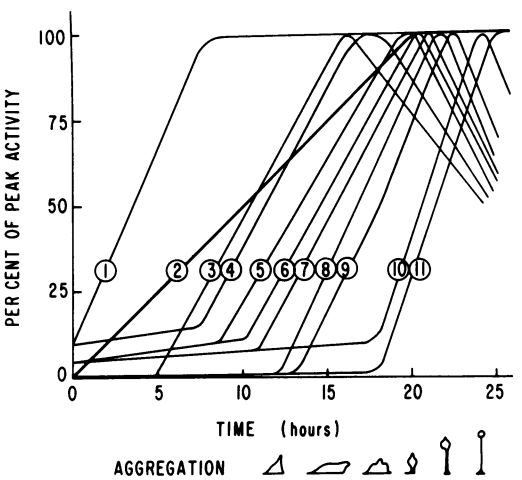

FIG. 8. *Developmentally controlled enzymes of D. discoideum. The kinetics of accumulation of eleven enzymes are presented as per cent of peak activity along with the stages in morphogenesis. No. 1, acetylglucosaminidase, EC 3.2.1.30 (7); no. 2, α-mannosidase, EC 3.2.1.24 (this paper); no. 3, trehalose-phosphate synthetase, EC 2.3.1.15 (14); no. 4, threonine dehydrase, EC 4.2.1.16 (S. Pong and W. Loomis, manuscript in preparation); no. 5, tyrosine transaminase, EC 2.6.1.5 (S. Pong and W. Loomis, manuscript in preparation); no. 6, uridine diphosphoglucose pyrophosphorylase, EC 2.7.7.9 (14); no. 7, UDP-galactose polysaccharide transferase (14); no. 8, UDP-glucose epimerase, EC 5.1.3.2 (A. Telser and M. Sussman in press); no. 9, glycogen phosphorylase, EC 2.4.1.1 (R. Firtel and J. Bonner in press); no. 10, alkaline phosphatase, EC 3.1.3.1 (8); and no. 11, β-glucosidase-2, EC 3.2.1.2 (1).*

The accumulation of α-mannosidase requires concomitant protein synthesis (Fig. 4). Since the enzyme appears to be stable in the absence of protein synthesis, it is likely that the increase in specific activity reflects an increase in de novo synthesis of the enzymes. RNA synthesis required for this enzyme appears to preceed the synthesis of the enzyme by about 3 hr. A similar relationship between periods of RNA and protein synthesis has been found for acetylglucosaminidase (7), threonine dehydrase (Pong and Loomis, *manuscript in preparation*) and β-glucosidase (1). Different relationships have been found for several other developmentally controlled enzymes (8, 14).

Synthesis of α-mannosidase does not require intercellular interactions which result from aggregation of the amoebae, since synthesis is initiated before the aggregation stage and proceeds normally in mutant strains which are unable to aggregate. The observation that dissociation of wild-type pseudoplasmodia to single cells does not affect the accumulation of α-mannosidase supports this conclusion. Nevertheless, the period of synthesis of this enzyme must be tightly coupled to the developmental program since it is accelerated in the mutant strain (FR-17) in which all facets of cytodifferentiation occur precociously and is delayed in parallel with other differentiations in the slow mutant, GN-3. It appears that the mechanism which insures coordination of the various biochemical differentiations determines the period of synthesis of α-mannosidase. Although the rate of accumulation is also accelerated in strain FR-17 and slowed in strain GN-3, the magnitude of the change in rate is not sufficient to compensate for the altered periods of synthesis in these strains. Thus strain FR-17 only accumulates approximately 75% of the normal amount of α-mannosidase, whereas strain GN-3 accumulates about 30% more enzyme than the wild type. It seems that temporal and quantitative controls of α-mannosidase are at least partially independent.

Like other acid hydrolases of *D. discoideum*, α-mannosidase is localized in lysosomes during development. These compartments may serve an autophagic function to provide energy for cell movement as well as subunits for the synthesis of new macromolecules (R. George, Ph.D. Thesis, University of Hawaii, 1968). α-Mannosidase may

serve in this degratory role. However, another possibility is that this enzyme is involved in the alteration of the mannose-containing polysaccharide so as to allow the continued synthesis and excretion of mucopolysaccharide. Only the isolation of a mutation in the structural gene for α-mannosidase would permit an unequivocal determination of the physiological role of α-mannosidase.

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