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# Acetylglucosaminidase, an Early Enzyme in the Development of *Dictyostelium discoideum*

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The specific activity of acetylglucosaminidase has been found to increase more than 10-fold during the first 10 hr of development in the cellular slime mold *Dictyostelium discoideum*. The specific activity then remained essentially constant until after germination. The activity was purified 36-fold and found to behave as a single protein species. The increase in specific activity required concomitant protein synthesis. If ribonucleic acid synthesis was preferentially inhibited during the period of synthesis of acetylglucosaminidase, further increase in enzymatic activity stopped after 2 hr. The increase in activity did not occur in a mutant strain which did not undergo the first step in morphogenesis. Mutant strains, blocked slightly later in morphogenesis, synthesized the enzyme at the normal rate but for an extended period. It was concluded that the initiation and termination of synthesis of acetylglucosaminidase are controlled by the developmental program.

It has been thought for many years that development of multicellular organisms may result from differential gene activation (10). Changes in the specific activity of enzymes during development can give evidence on differential gene action when the relation to protein and ribonucleic acid (RNA) synthesis is determined.

The development of the cellular slime mold *Dictyostelium discoideum* presents a favorable system for genetic and biochemical studies, because large numbers of cells can be induced to undergo multicellular development synchronously (16) and techniques for clonal isolation of mutants with altered developmental capacities have been perfected (24). The specific activity of a large number of enzymes does not change appreciably during the development of *D. discoideum* (3, 23). However, three enzymes have been found which are synthesized only during discrete periods of slime-mold development and which require prior RNA synthesis: trehalose-phosphate synthetase (EC 2.3.1.15) activity increases during the pseudoplasmodial stage (14), and uridine diphosphate (UDP)-glucose pyrophosphorylase (EC 2.7.7.9) and UDP-galactose polysaccharide transferase activities increase when the pseudoplasmodia culminate to form spores and stalk cells (1, 18, 20).

This study concerns an enzyme activity which increases by more than 10-fold early in slime-mold development during the period of aggregation prior to multicellular organization. The increase in specific activity requires concomitant protein synthesis and previous RNA synthesis.

## MATERIALS AND METHODS

**Chemicals.** *p*-Nitrophenyl derivatives of β-D-*N*-acetylglucosamine, β-D-glucuronide, β-D-glucoside, α-D-glucoside, β-D-xylopyranoside, and *o*-nitrophenyl β-D-galactoside were purchased from Calbiochem. *p*-Nitrophenyl β-D-*N*-acetylgalactosamine was purchased from the Cyclo Chemical Corp. (Los Angeles, Calif.). Cycloheximide (Acti-Dione) was purchased from The Upjohn Co. (Kalamazoo, Mich.). Actinomycin D was a gift from Merck Co., Inc. (Rahway, N.J.).

Membrane filters were purchased from the Millipore Filter Corp. (Bedford, Mass.).

**Chromatographic methods.** Carbohydrates were separated on Whatman no. 1 paper with ascending solvent systems either of *t*-butyl alcohol-methyl ethyl ketone-formic acid-water (8:6:3:3) or of isopropyl alcohol-water-HCl (65:18.4:16.6; reference 4). The sugars were located by use of an $Ag(NO_3)$ stain.

**Organism.** *D. discoideum* strain NC-4 (haploid) and strain KY-3 (the morphological mutant) have been previously described (24). Five strains which fail to aggregate normally (VA-3 to VA-7) were isolated from strain NC-4 by Diane Van Alstyne who used the method of Yanagawa, Loomis, and Sussman (24). Two other strains which fail to aggregate (min 4 and min 5) were isolated by Loomis and Ashworth (8).

The amoebae were grown in association with *Aerobacter aerogenes* (16). Development was initiated by removal of the bacteria and was allowed to proceed at

22 C on membrane filters (Millipore HABP 047) saturated with phosphate buffer salt solution (16).

**Acetylglucosaminidase assay.** Approximately $5 \times 10^7$ cells were collected from the membrane filters and suspended in 3 ml of distilled water. The samples were frozen, thawed, and treated for 30 sec on a Branson Sonifier. Assays on crude extracts were performed immediately with 20 to 200 $\mu$g of protein per ml in $10^{-2}$ M acetate buffer, *p*H 5, which contained $8 \times 10^{-3}$ M *p*-nitrophenyl *N*-acetylglucosamine. After incubation at 35 C for 5 to 100 min, the reaction was stopped by the addition of an equal volume of 1 M $Na_2CO_3$. Substrate-dependent absorption at 420 nm was determined with a Zeiss spectrophotometer. A unit of activity is defined as the amount which will liberate 1 nmole of *p*-nitrophenol per min under the above conditions. Specific activity is expressed as units per milligram of protein. Protein was determined by the method of Lowry et al. (9).

**Acetylglucosamine determinations.** The concentration of *N*-acetylglucosamine was estimated by the method of Reissig, Strominger, and Leloir (12) with *p*-dimethylaminobenzaldhyde reagent.

## RESULTS

**Characteristics of the reaction.** The activity of acetylglucosaminidase (EC 3.2.1.30) was estimated by observation of the increase in optical density at 420 nm after incubation of the extract with *p*-nitrophenyl *N*-acetyl $\beta$-D-glucosamine. The reaction product had an absorption spectrum from 310 to 500 nm, which is identical with authentic *p*-nitrophenol. When 1 $\mu$ mole of substrate was totally hydrolyzed by incubation with partially purified enzyme (600 units/mg of protein), the only observable reducing compound cochromatographed with authentic *N*-acetylglucosamine in two solvent systems. It was concluded that the enzyme activity under study catalyzes the following reaction: *p*-nitrophenyl *N*-acetylglucosamine + $H_2O \rightarrow$ *p*-nitrophenol + *N*-acetylglucosamine.

The formation of *p*-nitrophenol is linear with respect to time up to 100 min, and linear with respect to amount of extract up to 0.5 mg of protein per ml at 35 C until more than 0.1 $\mu$mole has been formed. The Michaelis constant ($K_m$) for *p*-nitrophenyl *N*-acetyl $\beta$-D glucosamine is $10^{-3}$ M under standard assay conditions. The enzyme has a broad *p*H optimum in the range *p*H 4.5 to 5.5. It is stable to freezing and thawing; the activity is stable for more than 10 min at 50 C, but it is inactivated by 5 min at 80 C. The rate of the reaction is linearily dependent on temperature from 20 to 45 C and shows a $Q_{10}$ of 2 in this range.

**Partial purification of acetylglucosaminidase.** Approximately $5 \times 10^9$ amoebae of *D. discoideum* strain NC-4 were allowed to develop on membrane supports for 16 hr before they were collected in $10^{-3}$ M phosphate buffer, *p*H 6. The cells were concentrated by slow-speed centrifugation, and the pellet was resuspended in buffer. All activity was found associated with the cells. After it was frozen, thawed, and sonically treated, the extract was made 0.5% in streptomycin sulfate. The supernatant liquid, after centrifugation at $3,000 \times g$ for 15 min, was brought to 60% saturation with ammonium sulfate. After 2 hr at 0 C, the precipitate was collected by centrifugation and was found to contain 85% of the acetylglucosaminidase activity present in the starting material.

Material, partially purified by the above steps, was chromatographed on a diethylaminoethyl (DEAE)-Sephadex column that was equilibrated with 0.2 M NaCl, $10^{-4}$ M phosphate buffer (*p*H 6), and eluted by an NaCl gradient from 0.2 M to 0.7 M. The enzyme eluted as a single peak at about 0.4 M NaCl (Fig. 1). This procedure resulted in a 36-fold purification of the enzyme. The activity was found to be excluded by Sephadex G100. At no time during the purification was there evidence for more than a single molecular species with acetylglucosaminidase activity.

The kinetics, *p*H optimum, and temperature

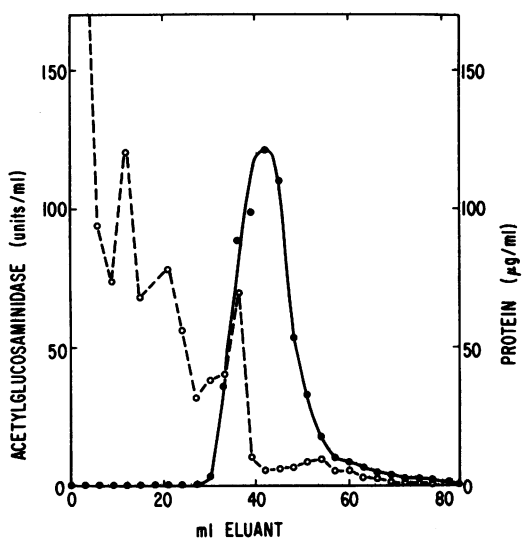


FIG. 1. *Purification of acetylglucosaminidase. Acetylglucosaminidase (1,500 units), purified to a specific activity of 320 units/mg of protein from a crude extract of D. discoideum pseudoplasmodia, was applied to a column (1 by 10 cm) of DEAE-Sephadex in $10^{-4}$ M potassium phosphate buffer, pH 6, containing 0.2 M NaCl. The enzyme was eluted with a linear gradient of NaCl from 0.2 M to 0.7 M in 100 ml of the phosphate buffer. Samples (3 ml) were collected and assayed for protein* (○) *and acetylglucosaminidase* (●).

characteristics of the reaction catalyzed by the partially purified enzyme were similar to those catalyzed by the crude extract; this suggests that no diffusable cofactors, activators, or inhibitors of the enzyme are present in crude extract. The specificity of the partially purified material was tested on a variety of compounds (Table 1). Only *p*-nitrophenyl *N*-acetylglucosamine and *p*-nitrophenyl *N*-acetylgalactosamine were hydrolyzed to a significant extent under standard assay conditions. A highly purified sample of acetylglucosaminidase from pinto beans also catalyzes the hydrolysis of *p*-nitrophenol galactosamine (2).

**Kinetics of enzyme synthesis during development.** When the food supply of *D. discoideum* is depleted or removed, the solitary vegetative amoebae aggregate over a period of several hours into masses of about $10^5$ cells. During the next few hours, these masses organize into migratory pseudoplasmodia. After an additional period of about 8 hr, the pseudoplasmodia settle down on the support and form tall gently tapering stalks. At the top of each stalk, about $5 \times 10^4$ cells encapsulate to form the spores (legend, Fig. 2). This developmental sequence occurs in the absence of exogenous food supply or significant cell division (11, 21).

Samples were taken for determination of acetylglucosaminidase activity at various times after initiation of development (Fig. 2). The specific activity of acetylglucosaminidase increases by a factor of about 10 during the first 8 to 10 hr of development; it then remains essentially constant. The gradual rise in specific activity during the latter part of development may be due to preferential protection from protein catabolism rather than enzyme synthesis since total protein per cell decreases considerably during that period (5, 22).

TABLE 1. *Substrate specificity of acetylglucosaminidase*

| Compound[a] | Relative reaction rate |
|---|---|
| *p*-Nitrophenyl *N*-acetyl β-D-glucosamine | 100 |
| *p*-Nitrophenyl *N*-acetyl β-D-galactosamine | 46 |
| *o*-Nitrophenyl β-D-galactoside | 0.2 |
| *p*-Nitrophenyl β-D-glucuronide | <0.1 |
| *p*-Nitrophenyl β-D-glucoside | <0.1 |
| *p*-Nitrophenyl β-D-xylopyranoiside | <0.1 |
| *p*-Nitrophenyl α-D-glucoside | 0.1 |
| Hyaluronic acid | <0.1 |
| Chitin (poly *N*-acetylglucosamine) | <0.1 |

[a] Concentration in reaction mixture was: *p*-nitrophenyl *N*-acetyl β-D-glucosamine and *p*-nitrophenyl *N*-acetyl β-D-galactosamine, $8 \times 10^{-3}$M; hyaluronic acid, 5 mg/ml; chitin, 15 mg/ml; and all other compounds at $10^{-2}$M. Reaction with hyaluronic acid and chitin was assayed by estimation of release of *N*-acetylglucosamine; reaction with other compounds was assayed by increase in absorbance at 420 nm. All compounds were incubated with at least 10 units of partially purified enzyme (specific activity, 6,000) up to 1 hr at *p*H 5 and 35 C.

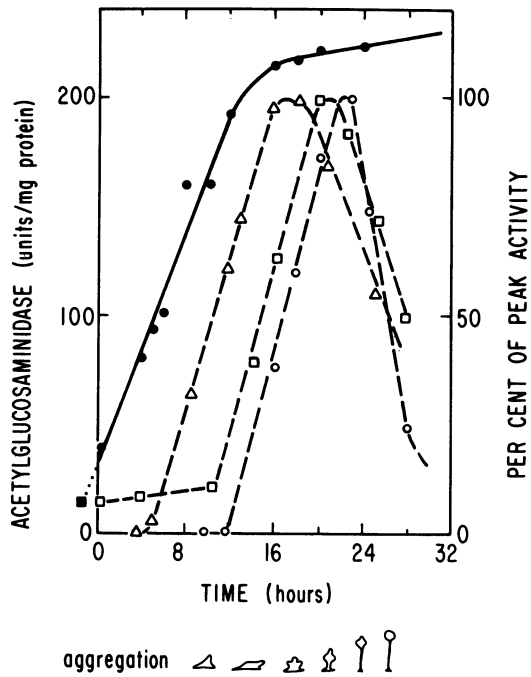


FIG. 2. *Kinetics of accumulation of acetylglucosaminidase. Cells of D. discoideum were collected from the membrane filters at various times after the initiation of development and the specific activity of acetylglucosaminidase was determined* (●). *A single determination on vegetative amoebae growing exponentially was performed* (■). *Kinetics of accumulation of other developmentally controlled enzymes are from the data of Roth et al. (14) and are presented as per cent of peak activity: trehalose-phosphate synthetase* (△), *UDP-glucose pyrophosphorylase* (□), *UDP-galactose polysaccharide transferase* (○). *Schematic representation of morphogenesis in D. discoideum is presented with the time scale.*

When samples taken at 0, 12, and 24 hr of development were mixed in all possible combinations, the activities were strictly additive; this suggests that no diffusable inhibitors or activators can account for the difference in specific activity in the different samples.

Cycloheximide has been shown to inhibit protein synthesis preferentially more than 85% within 15 min in *D. discoideum* (1, 15). When protein synthesis was blocked at different times during the

aggregation of the amoebae, the accumulation of acetylglucosaminidase ceased, while the activity which was present appeared stable (Fig. 3). It was concluded that the majority of the increase in specific activity requires concomitant protein synthesis.

Actinomycin D has been shown to inhibit RNA synthesis preferentially in *D. discoideum* more than 95% within 30 min (17, 20). When this drug was added at 0 or 4 hr after the initiation of development, acetylglucosaminidase continued to accumulate for only about 2 hr; it then remained approximately constant (Fig. 4). It appears that RNA synthesis must occur during development for the normal amount of acetylglucosaminidase to accumulate. Enzyme-forming capacity, expressed in the absence of RNA synthesis, is never more than 20% of maximum activity. This finding is in contrast to results with two other developmentally controlled enzymes which continue to accumulate at the normal rate for 8 hr or more after RNA synthesis is inhibited (1, 20).

**Relationship of acetylglucosaminidase synthesis to morphogenesis.** Many morphogenetically aberrant mutants of *D. discoideum* have been described (19, 24). Seven strains which fail to aggregate normally were independently isolated after treatment with nitrosoguanidine. All but one of these strains accumulated acetylglucosaminidase to a specific activity higher than that in wild-

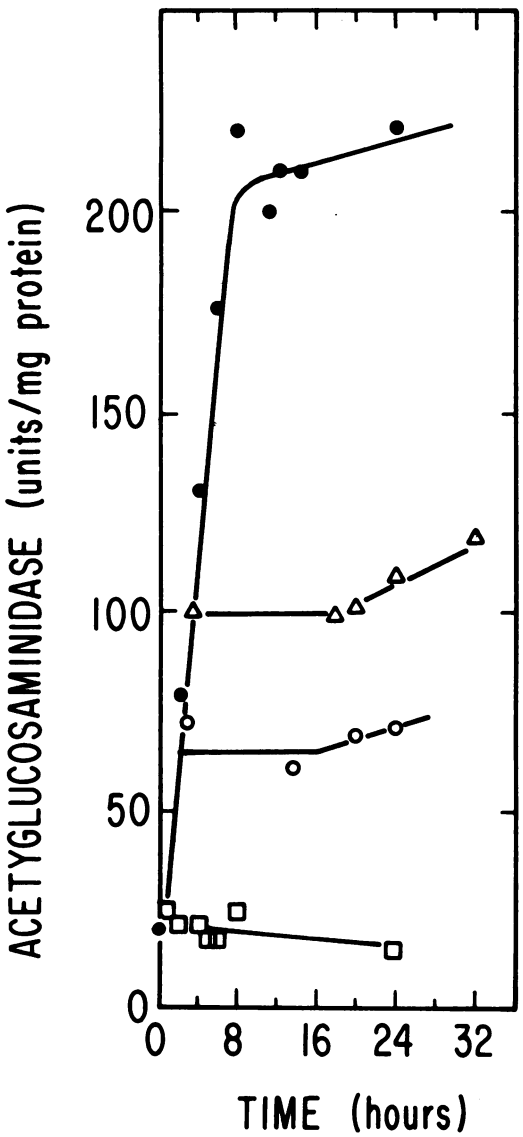


FIG. 3. *Inhibition of accumulation of acetylglucosaminidase by cycloheximide. Membrane filters with developing amoebae were transferred to pads containing cycloheximide (500 μg/ml) at 0 (□), 2 (○), 4 (△) hr after the initiation of development. Control cells were not transferred (●).*

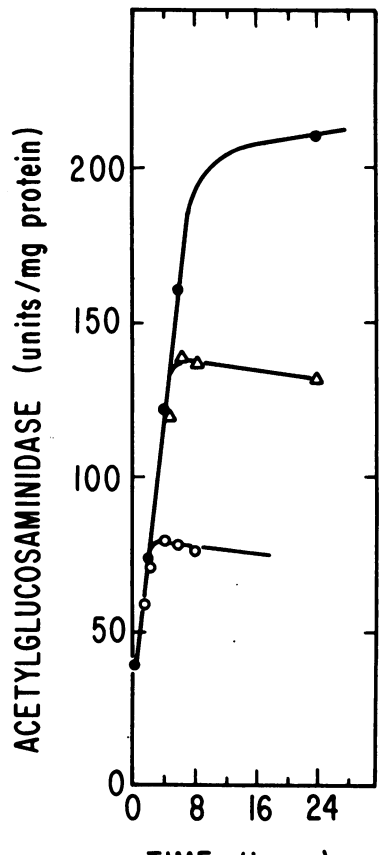


FIG. 4. *Inhibition of accumulation of acetylglucosaminidase by actinomycin D. Membrane filters with developing amoebae were transferred to pads containing actinomycin D (100 μg/ml) at 0 (○) and 4 (△) hr. Control cells were not transferred (●).*

type cells after the initiation of development. The exceptional strain, VA-5, showed no sign of aggregation and failed to synthesize significant amounts of the enzyme (Fig. 5). Thus, an early morphological aberration affects synthesis of acetylglucosaminidase, but completely normal aggregation does not appear to be required for the synthesis of this enzyme.

The increase in specific activity of acetylglucosaminidase in the six semi-aggregateless strains (min 4, min 5, VA-3, -4, -6, and -7) does not stop after 8-hr development, as in wild-type cells (Fig. 5). To determine whether continued enzyme synthesis in one of these strains, VA-4, requires continued RNA synthesis, actinomycin D was added to cells of this strain at 0 and 8 hr of development. Samples were collected at 8, 20, and 34 hr for determination of acetylglucosaminidase activity. The specific activity increased for less than 2 hr in cells treated with the drug, while previously accumulated activity remained constant. It is concluded that continuous RNA synthesis is required for the prolonged enzyme synthesis in this semi-aggregateless strain.

Strain KY-3 is a morphological mutant which forms pseudoplasmodia but fails to culminate (24). Accumulation of acetylglucosaminidase in this strain follows kinetics similar to those in the wild-type strain (Fig. 4).

**Absence of spatial localization.** During the development of *D. discoideum*, cells of the initially homogeneous population of amoebae differentiate to either of the two terminal forms, spores or stalk cells. To determine whether acetylglucosaminidase activity is restricted to one or the other of these cell types in mature fruiting bodies, spores and stalk cells were separated by micromanipulation from a 24-hr preparation. There was no significant difference in the specific activity of acetylglucosaminidase in these cell types (Table 2).

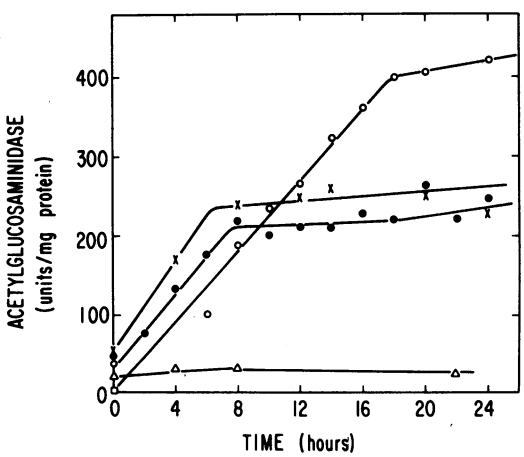


FIG. 5. *Acetylglucosaminidase in morphologically aberrant mutants of D. discoideum: strain VA-5, △; VA-4, ○; KY-3, ×; NC-4 wild type, ●. A single determination of vegetative cells of strain VA-4 growing exponentially was performed (□).*

## DISCUSSION

The increase in specific activity of acetylglucosaminidase reported here is a facet of the development of *D. discoideum*. The increase does not occur in a mutant strain that is unable to accomplish the first step in morphogenesis (aggregation), but it does occur in strains which form imperfect aggregates. The termination of enzyme synthesis appears to be another facet of the development of this organism, since a mutation which affects aggregation also affects termination of accumulation of acetylglucosaminidase.

The increase in activity does not appear to be an artifact of the assay or the result of diffusable activations or inhibitors. Concomitant protein synthesis is required for accumulation of activity, a result which suggests that the increase in specific activity may result from de novo synthesis of the enzyme. In support of this view is the observation that concomitant RNA synthesis must also occur for the majority of the activity to accumulate. Synthesis of messenger RNA molecules has been shown by similar methods to be required for de novo enzyme synthesis in bacteria (7, 19).

A requirement for RNA synthesis during development of *D. discoideum* has been shown for the synthesis of three other developmentally controlled enzymes (13). The full complement of enzyme-forming capacity for two of these enzymes is stable for at least 6 hr in the absence of RNA synthesis (13). Enzyme-forming capacity for the third enzyme, trehalose-phosphate synthetase, like that for acetylglucosaminidase, decays within 2 hr in the absence of RNA synthesis. Thus, protein synthesis in *D. discoideum* appears to depend on both stable and unstable RNA species.

TABLE 2. *Specific activity of acetylglucosamidase in various cell types*

| Cell type | Acetylglucosaminidase[a] |
|---|---|
| *Aerobacter aerogenes* | <2 |
| Vegetative amoebae | 16 |
| Mature fruiting body | 210 |
| Spore cells | 218 |
| Stalk cells | 194 |

[a] Assays were performed as described in Materials and Methods. Values are expressed as units per milligram of protein.

Unlike the three previously described developmentally controlled enzymes, acetylglucosaminidase is stable throughout morphogenesis. The specific activity decreases to that of vegetative cells only after germination of the spores.

It must be emphasized that the physiological role of acetylglucosaminidase is unknown. However, the primary purpose of these studies is to define the product of a single gene under developmental control; acetylglucosaminidase appears to be such a molecule. Studies on the factors which control the formation of this enzyme are in progress.

## ACKNOWLEDGMENTS

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