Properties and Developmental Regulation of an α -D-Galactosidase from *Dictyostelium discoideum*

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An α -D-galactosidase was detected in cells of the cellular slime mould, *Dictyostelium discoideum*, at all stages of development. Its specific activity was highest during early development (interphase), and this accumulation of enzyme appears to require protein synthesis *de novo*. Its subcellular distribution differs from that of other *D. discoideum* glycosidases, since most activity was recovered in the soluble fraction. No evidence was obtained for more than one isoenzymic form after subjection of extracts to electrophoresis and various chromatographic procedures. It is excreted from the cell during development, but no evidence was found for an extracellular function for the enzyme.

Dictyostelium discoideum, a cellular slime mould, has many advantages as a model system for the study of eukaryotic development (Loomis, 1975). Its life-cycle can be divided into two mutually exclusive phases, vegetative growth and development. Free-living myxamoebae grow and divide until they use up most of their bacterial food source (vegetative stage). After a period of several hours (interphase), the cells come together to form mounds visible to the eye (aggregation), which topple over to migrate across the supporting surface (slug stage). At the end of migration, the cell mass rounds up again (culmination), and a sorocarp is raised into the air on a narrow cylinder of non-viable cells (fruiting body formation). The fruiting body consists mainly of spores and stalk cells, which are morphologically and biochemically distinct, both from each other and from myxamoebae.

Carbohydrates are thought to play an important role in the development of *D. discoideum*, the amount of carbohydrate being maintained against a marked decrease in protein and RNA (White & Sussman, 1961). Most attention has been given to the metabolism of glucose-containing compounds, but the metabolism of galactose is also of considerable interest for several reasons. An acid mucopolysaccharide containing galactose is formed during terminal differentiation and this ultimately accounts for 1–2% of the dry weight of the fruiting body (White & Sussman, 1963). There are dramatic increases in the specific activities of UDP-galactose polysaccharide transferase (Sussman & Osborn, 1964) and UDP-galactose epimerase (Telser & Sussman, 1971), reaching peak values during culmination. These enzyme accumulations absolutely require cellular interaction, and in the case of UDP-galactose epimerase

will not begin until the cessation of slug migration no matter how long the migratory period is prolonged (Newell, 1971). An agglutinin or lectin that specifically recognizes sugars of a galactose-type configuration has been detected in homogenates of *D. discoideum* and has developmental kinetics similar to those of aggregation-specific contact sites (Rosen *et al.*, 1973). The agglutinin, discoidin (Simpson *et al.*, 1974) or discoidin I (Frazier *et al.*, 1975), is located on the cell surface (Chang *et al.*, 1975), and can agglutinate amoebae by binding to receptor sites that are also developmentally regulated (Reitherman *et al.*, 1975). Finally, we have found that galactose is one of the most abundant sugars recovered after acid hydrolysis of plasma-membrane 'ghosts' prepared from *D. discoideum* (D. C. Kilpatrick & J. L. Stirling, unpublished work).

The turnover of galactose-containing polysaccharides during the development of *D. discoideum* is likely to involve α - and β -galactosidases, but very little work has been done on these enzymes, although there have been detailed reports of several other glycosidases, including β -N-acetyl-D-glucosaminidase (Loomis, 1969a), β -D-glucosidase (Coston & Loomis, 1969) and α -D-mannosidase (Loomis, 1970). Every & Ashworth (1973) made a detailed study of extracellular glycosidases of *D. discoideum*; they were unable to detect any α -D-galactosidase, and limited their study of β -D-galactosidase, because it was found to be unstable.

Those and other published studies concerning *D. discoideum* glycosidases used *p*-nitrophenyl glycosides in their assays of enzyme activity. We have studied the properties of several *D. discoideum* glycosidases by using 4-methylumbelliferyl substrates, which permit a more sensitive fluorogenic assay. Here we report the properties and developmental regulation of the previously undetected α -D-galactosidase.

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Materials and Methods

Materials

All 4-methylumbelliferyl glycosides, 4-methylumbelliferyl phosphate and galactono- γ -lactone were from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Yeast extract, Bacto-Agar and Bacto-Peptone were from Difco Laboratories, Detroit, MI, U.S.A. Cycloheximide, actinomycin D from *Streptomyces chrysomallus* and bovine serum albumin (type F) were from Sigma (London) Chemical Co., Kingston, Surrey, U.K. Daunorubicine hydrochloride (daunomycin) was from Calbiochem, La Jolla, CA, U.S.A. Sephadex and concanavalin A–Sepharose were from Pharmacia (G.B.), London W.5, U.K. Triton X-100 and α -methyl glucoside were from BDH, Poole, Dorset, U.K. DEAE-cellulose was from W. R. Balston, Maidstone, Kent, U.K. Hydrolysed starch was from Connaught Medical Research Laboratories, Toronto, Canada. Galactose, glucose, sucrose and all common laboratory chemicals were from Fisons Scientific Apparatus, Loughborough, Leics., U.K. We are grateful to Professor J. Ashworth for a gift of spent growth medium of the axenic mutant of *D. discoideum* Wag 8.

Preparation of buffers

In all cases the pH was measured with a Pye model 78 pH-meter calibrated with appropriate standard buffers. Sodium glycinate buffer, pH10.4, was prepared by titrating 0.5M-glycine with 0.5M-NaOH. Sodium citrate/phosphate buffer, pH4.5, used for enzyme assays only, was prepared by titrating 0.15M-citric acid with 0.15M- Na_2HPO_4 . For all other purposes, including the determination of pH-activity curves, glycine/HCl buffers (0.1M-glycine titrated with 0.1M-HCl) were used for the pH range 2–3, sodium citrate/phosphate buffers (0.1M-citric acid titrated with 0.2M- Na_2HPO_4) for the pH range 3–5.5, and sodium phosphate buffers (0.1M- NaH_2PO_4 titrated with 0.1M- Na_2HPO_4) were used for the pH range 5.5–8.

Preparation of cell extracts

D. discoideum cells were broken by using an MSE 150W Ultrasonic Disintegrator. Homogenates of amoebae were prepared by sonication for 1 min at full power, resulting in over 95% cell breakage. Culminating cells were treated for 5 min at full power with similar results. Fruiting bodies were treated for 45–60 min at full power; this resulted in about 50% breakage of spores and almost total rupture of stalk cells. Sonication took place in glass tubes kept cool by immersion in a beaker of ice. Disruption of fruiting bodies by prolonged sonication did not seem to result in appreciable denaturation of α -D-galactosidase, since

similar activities were recovered from cells disrupted in a French press.

Enzyme assays

α -D-Galactosidase was assayed by incubating 10 μ l of sample containing enzyme with 50 μ l of 5 mM-4-methylumbelliferyl α -D-galactoside in citrate/phosphate buffer, pH3.5, for 30 min at 37°C. The reaction was stopped by the addition of 1.0 ml of sodium glycinate, pH10.4. β -D-Galactosidase, acid phosphatase and β -N-acetyl-D-hexosaminidase were assayed by incubating 1.0 ml of an appropriate dilution of the sample with 1.0 ml of 2 mM-4-methylumbelliferyl β -D-galactoside or 2 mM-4-methylumbelliferyl phosphate or 0.4 mM-4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucoside in sodium citrate/phosphate buffer, pH4.5, at 37°C. Incubation times of 30–60 min for β -D-galactosidase, 30 min for acid phosphatase and 15 min for β -N-acetyl-D-hexosaminidase were found to be suitable, the reaction being stopped by the addition of 3.0 ml of sodium glycinate buffer, pH10.4. The 4-methylumbelliferone liberated in the course of these assays was measured by using a Locarte fluorimeter (activation at 350 nm, measurement above 440 nm), standardized by using a solution of quinine sulphate (4 mg/litre in 0.05M- H_2SO_4). Absolute enzyme activity was estimated from a calibration curve after subtraction of the substrate blank.

To determine specific activities, protein content was measured by the method of Lowry *et al.* (1951), modified by using trisodium citrate in place of sodium tartrate. Bovine serum albumin was used as a protein standard.

Cultivation of micro-organisms

Dictyostelium discoideum NC-4 was grown in association with *Escherichia coli* B/r on nutrient-agar plates as described by Sussman (1966). Synchronous development was induced by separating vegetative amoebae from bacteria by differential centrifugation (Sussman, 1966), and placing 0.5 ml of a suspension of amoebae on to the surface of a membrane filter (Millipore Corp.; 0.8 μ m pore size), supported by an absorbent pad saturated with buffered salts solution (Sussman, 1966), subsequently referred to as lower-pad solution. During growth and development, amoebae were maintained at 22–23°C in a cooled incubator (Gallenkamp). Water was present to ensure a high humidity.

To study the effect of inhibitors on development or enzyme accumulation, the agent to be used was dissolved in the lower-pad solution.

Determination of kinetic properties

The dependence of enzyme activity on pH was determined by placing enzyme solution in an appropriate buffer, and starting the reaction by the addition

of an aqueous solution of substrate to bring the final concentration of substrate to that of the standard assay. Substrate blanks were used at each pH.

Thermal-stability measurements were carried out by preincubating enzyme samples at a controlled pH and constant temperature for various times before carrying out the standard enzyme assay.

Michaelis (K_m) and inhibitor (K_i) constants were estimated as described by Dixon (1953).

Partial purification of α -D-galactosidase on DEAE-cellulose

Vegetative cells and aggregates were separately sonicated, centrifuged (87000g for 80min), and the supernatant of each (3.0ml) applied separately to a column (4.5cm $\times$ 1.25cm diam.) of DEAE-cellulose equilibrated with 5mM-sodium phosphate, pH6.5. The first nine (4.6ml) fractions were eluted (flow rate 35ml/h) with the same buffer before a linear NaCl gradient was begun, the concentration of NaCl reaching 0.35M in 300ml. All the α -D-galactosidase was retained by the column when applied under these conditions and was eluted in the salt gradient. α -D-Galactosidase from vegetative cells was purified 8-fold with 89% recovery; α -D-galactosidase from aggregates was purified 12-fold with 71% recovery.

Partial purification of α -D-galactosidase on concanavalin A-Sepharose

Large aggregates (approx. 8×10^8 cells) and vegetative amoebae (approx. 5×10^8 cells) were separately collected, washed in 5mM-sodium phosphate, pH6.8, containing 0.1M-NaCl (salt/phosphate buffer), sonicated, then centrifuged for 80min at 87000g in an MSE 25 centrifuge refrigerated at 4°C. The soluble homogenates so obtained were each applied under gravity to a column (3.5cm $\times$ 1.6cm diam.) of concanavalin A-Sepharose equilibrated with salt/phosphate buffer at 4°C. The concanavalin A-Sepharose was washed with salt/phosphate buffer (12 column volumes) at a flow rate of 40ml/h. The columns were then removed from the cold laboratory, allowed to reach room temperature (approx. 20°C), then eluted under gravity with 3 column volumes of 1M- α -methyl glucoside in salt/phosphate buffer at room temperature in order to displace the bound α -D-galactosidase.

After dialysis (4 $\times$ 8 litres) against salt/phosphate buffer over 24h, and further (3h) dialysis against 4 litres of water at 4°C, the eluates were adjusted to 65% saturation with $(\text{NH}_4)_2\text{SO}_4$, and stirred overnight at 4°C. The precipitates were collected by centrifugation (30000g, 1h), re-dissolved in salt/phosphate buffer, and dialysed first against salt/phosphate buffer overnight then water for 3h.

α -D-Galactosidase from vegetative (aggregating) cells was purified 4-fold on concanavalin A-Sepha-

rose, and $(\text{NH}_4)_2\text{SO}_4$ precipitation brought the purification to 5-fold, whereas the enzyme from aggregating cells was purified 5.5-fold, and 8-fold respectively.

Gel filtration

The soluble homogenate (3.0ml) of vegetative or aggregating cells was applied to a column (29cm $\times$ 2.6cm) of Sephadex G-150 equilibrated with 5mM-sodium phosphate, pH6.5. Fractions (2.9ml) were collected during elution with the same buffer at a flow rate of 20ml/h.

Subcellular fractionation

Amoebae were grown on agar plates until the bacterial lawn was being visibly consumed and some aggregation was taking place. The cells were removed in cold water, washed free of bacteria in cold water, then washed in cold 0.25M-sucrose and resuspended at a concentration of approx. 10^8 cells/ml. The suspension (10ml) was placed in a Teflon/glass homogenizer and the pestle rotated with an electric stirrer (Anderman and Co., London S.E.1, U.K.) at full speed. After 15 strokes, about 40% cell breakage had occurred, judged by counting in a haemocytometer. The suspension was then fractionated as described by Ashworth & Quance (1972) to give a nuclear fraction containing unbroken cells (which was discarded), a lysosomal pellet, a microsomal pellet and a residual soluble fraction. Both pellets were resuspended in cold 0.25M-sucrose. Triton X-100 was added to each fraction to a final concentration of 0.1% before assay for enzyme activities.

Electrophoresis

Electrophoresis in starch gel (Smithies, 1955) was carried out by using 11% hydrolysed starch in 5.3mM-sodium phosphate, pH7.0, with 40mM-sodium phosphate in the buffer compartments. The sample was absorbed on to a filter-paper wick and inserted into the gel, which was placed in a refrigerated cabinet. The current was maintained at 40mA (220–250V) for 3h. After electrophoresis, enzyme activity on the cut gel surfaces was detected by incubation with 4-methylumbelliferyl α -D-galactoside by a method similar to that of Robinson *et al.* (1967).

Results

Detection of α -D-galactosidase

By using the fluorogenic assay based on the hydrolysis of 4-methylumbelliferyl α -D-galactoside, α -D-galactosidase was readily detected in extracts from all stages of development of the wild-type strain NC-4,

Fig. 1. pH-activity curve of α -D-galactosidase

The dependence of α -D-galactosidase activity on pH was determined by using glycine/HCl buffers for the pH range 2–3, citrate/phosphate buffers for the range 3–5.5, and phosphate buffers for the pH range 5.5–8. The values in this Figure were obtained by using a crude homogenate of vegetative amoebae, but similar profiles were obtained with a crude homogenate of aggregating amoebae and partially purified preparations from both vegetative and aggregating cells after chromatography on DEAE-cellulose. For further details see the Materials and Methods section.

and in the spent growth medium of an axenic mutant, Wag 8. α -D-Galactosidase was also detected in the homogenate of spores that had been removed from fruiting bodies by lightly brushing several sorocaps with a drop of water carried in a loop of platinum wire.

β -D-Galactosidases and other glycosidases also occurred in each of those extracts, but subsequent separation procedures clearly demonstrated that α -D-galactosidase activity belongs to a molecular species different from any of the glycosidases previously described.

Kinetic properties

α -D-Galactosidase was only appreciably active under acid conditions, with an optimum at pH 4.0 (Fig. 1). β -D-Galactosidase also displayed a pH-activity dependence typical of acid hydrolases, with highest activity between pH 3.5 and 4.5.

Both α - and β -galactosidases were competitively inhibited by galactose and by galactono- γ -lactone. α -D-Galactosidase was more sensitive to inhibition by galactose than to the lactone, although the reverse was true of the β -D-galactosidase (Table 1). It is noteworthy that the latter was relatively insensitive to the natural β -galactoside, lactose. Even with 100 mM lactose in the standard assay mixture, 90% of the activity of the lactose-free control was measured.

Table 1. Some kinetic constants of *D. discoideum* galactosidases

Michaelis constants (K_m) and inhibitor constants (K_i) were determined by the method of Dixon (1953). Supernatants obtained by centrifuging sonicated cells at 87000g for 80 min were used as a source of enzyme in these experiments.

Enzyme	K_m (mM)	K_i (mM)	
		Galactose	Galactono- γ -lactone
α -D-Galactosidase	1.8	2.0	31.5
β -D-Galactosidase	0.5	5.3	1.4

Electrophoretic and chromatographic behaviour of α -D-galactosidase

Several enzyme activities of *D. discoideum* are known to exist as isoenzymes, the relative proportions of which vary during development (Loomis, 1975). In a preliminary experiment to investigate the possibility of isoenzymic forms of α -D-galactosidase, samples from all stages of the life-cycle were subjected to electrophoresis in starch gel buffered at pH 7.0. α -D-Galactosidase was detected as a single band that migrated the same distance towards the anode irrespective of the stage of development.

After a soluble homogenate of vegetative amoebae was applied to a column of Sephadex G-150, the α -D-galactosidase activity was eluted as a single peak, and the profile and elution volume was similar when a soluble homogenate of aggregating cells was used.

On Sephadex G-200, α -D-galactosidase was eluted in the same volume as β -N-acetyl-D-hexosaminidase. Every & Ashworth (1973) estimated the mol.wt. of β -N-acetyl-D-hexosaminidase to be 170000 on Sephadex G-200, and from its behaviour α -D-galactosidase appeared to have a similar molecular weight. β -D-Galactosidase appeared to have a higher molecular weight, since it was eluted before these enzymes.

The soluble homogenates of vegetative and aggregating cells were each separately applied to a column of DEAE-cellulose equilibrated in phosphate buffer, pH 6.5, and the α -D-galactosidases from both were eluted as a single peak of activity at the same point (0.24 M-NaCl) in a linear NaCl gradient. β -D-Galactosidase activity was also eluted as a single peak at a lower NaCl concentration.

Thermal stability of the partially purified α -D-galactosidase

The α -D-galactosidase from vegetative cells partially purified on DEAE-cellulose was significantly more stable at 50°C (and pH 6.5) than was its counterpart from aggregating cells. Semi-logarithmic plots of

Table 2. Subcellular distribution of α -D-galactosidase

All activities are expressed as a percentage of that in the post-nuclear homogenate. The number in parentheses denotes the percentage activity when the pellet was assayed in buffered-sucrose/substrate to resist osmotic rupture of the lysosomes. For full experimental details see the Materials and Methods section.

	Enzyme		
	α -D-Galactosidase	β -N-Acetyl-D-hexosaminidase	Acid phosphatase
Lysosomal pellet	19.4 (14.5)	65.4	76.5
Microsomal pellet	1.7	1.5	0.8
Soluble fraction	78.0	44.3	29.9
Total	99.1	111.2	107.2

residual activity against time were linear for both preparations. The half-life of the vegetative enzyme was 10 min, and that of the aggregation enzyme was 3 min.

The α -D-galactosidase preparations after partial purification on concanavalin A-Sepharose and $(\text{NH}_4)_2\text{SO}_4$ fractionation from both vegetative and aggregating cells, however, were found to have a half-life of about 3 min at 50°C and pH 6.5. It is likely therefore that some stabilizing substance is present in the preparation of vegetative-cell α -D-galactosidase partially purified on DEAE-cellulose, which is not present in any of the other preparations. These measurements provide no evidence for more than one form of α -D-galactosidase, and suggest that the same enzyme is present during growth and development.

Subcellular distribution

The subcellular distribution of α -D-galactosidase was studied by using the method of Ashworth & Quance (1972). Acid phosphatase and β -N-acetyl-D-hexosaminidase were assayed for comparison, since they are known to be localized mainly in the lysosomal pellet. The α -D-galactosidase appears to be a cytoplasmic enzyme, since it was largely recovered in the soluble fraction under conditions in which acid phosphatase and β -N-acetyl-D-hexosaminidase were recovered in greatest abundance in the lysosomal fraction (Table 2). The behaviour of these markers is in good agreement with the experiments of Wiener & Ashworth (1970) and Ashworth & Quance (1972). The α -D-galactosidase activity recovered in the lysosomal pellet was less than 20% of the total activity in the post-nuclear homogenate. In another experiment, the lysosomal pellet was washed, and on that occasion 13% of the α -D-galactosidase activity of the post-nuclear homogenate was recovered. The lysosomal α -D-galactosidase was unusual in having an apparent latency (de Duve *et al.*, 1955) of only 25%, but the relationship between lysosomal and cytoplasmic α -D-galactosidase was not investigated.

Developmental kinetics

The variation in the specific activity of α -D-galactosidase during the development of *D. discoideum* on Millipore filters is shown in Fig. 2. The intracellular specific activity increased about 3-fold during the first few hours of development (interphase), then steadily declined. The specific activity of extracellular α -D-galactosidase recovered from the lower-pad solution increased steadily for 15 h, after which it remained constant.

The same samples were used to measure the specific activities of β -D-galactosidase and β -N-acetyl-D-hexosaminidase. The result of the β -D-galactosidase measurements are also illustrated in Fig. 2 for comparison. The intracellular specific activity of β -D-galactosidase increases very gradually throughout development, reaching its highest value in the fruiting body. Its extracellular specific activity possesses similar developmental kinetics to that of α -D-galactosidase. β -N-Acetyl-D-hexosaminidase underwent a 6-fold increase in intracellular specific activity during development, with similar kinetics to those described by Loomis (1969*a*) and Quance & Ashworth (1972) with *p*-nitrophenyl glycosides as substrates.

Effect of inhibitors of protein synthesis on α -D-galactosidase accumulation

To try to establish whether the increased specific activity of α -D-galactosidase during interphase was the result of protein synthesis *de novo*, measurements were made in the presence of inhibitors of RNA and protein synthesis over the first 5 h of development. When actinomycin D was present in the lower-pad solution, no significant difference was found between those cells and a control with respect to α -D-galactosidase accumulation. Cycloheximide, however, acted immediately to prevent the rise in α -D-galactosidase specific activity, and even appeared to cause a slight decrease (Fig. 3).

Firtel *et al.* (1973) have shown that in *D. discoideum* actinomycin D is more effective in inhibiting RNA

Fig. 2. Developmental kinetics of the galactosidases

Vegetative amoebae growing on agar plates in association with *E. coli* were washed free of bacteria and deposited (5×10^7 cells/filter) on Millipore filters to initiate development. At intervals, cells were washed off the filters in water and sonicated. The homogenate obtained was then assayed for enzyme activities and protein. The specific activity of α -D-galactosidase (●, ○) and β -D-galactosidase (■, □), expressed as nmol of substrate hydrolysed/min per mg of protein under standard assay conditions, is plotted against the time (h) after initiation of development. —, Intracellular activities; ----, extracellular activities.

synthesis if used with daunomycin. For that reason, a similar experiment was conducted in which actinomycin D was used in combination with daunomycin (Fig. 4). In the presence of the combined RNA-synthesis inhibitors, the rise in α -D-galactosidase specific activity was halted after 1 h.

Neither cycloheximide (0.5 mg/ml) nor daunomycin (250 μ g/ml) interfered with the assay of α -D-galactosidase.

Effect of inhibitors of galactosidases on development

The influence of galactose and galactono- γ -lactone on development was studied by allowing 10^8 vegetative amoebae to develop on Millipore filters saturated with lower-pad solution in which galactose or galactono- γ -lactone were dissolved.

Galactose retarded development in proportion to its concentration. Amoebae treated with 100 mM-galactose took about 1 h longer than the control to show visible aggregation, but apparently mature fruiting bodies had formed at the same time as the control. At higher galactose concentrations, the

amoebae took longer to form aggregates, and to form fruiting bodies, than corresponding control populations. With 500 mM-galactose, development took 32 h to complete instead of the normal 24 h.

Galactono- γ -lactone had a similar effect to that of galactose, but appeared to be a little more effective at retarding the developmental process. Fruiting-body formation had not taken place even after the amoebae had been incubated for 50 h when 500 mM-galactono- γ -lactone was used.

The effect of galactose and galactono- γ -lactone is probably not specific, since broadly similar results were also obtained by using glucose, *N*-acetylglucosamine and mannose in the same way.

Discussion

We have suggested that complex carbohydrates containing galactose are of considerable importance to the development and differentiation of *D. discoideum*. It was expected that both α - and β -galactosidases would be actively involved in carbohydrate turnover, since galactose in the form of

Fig. 3. Effect of actinomycin D and cycloheximide on α -D-galactosidase accumulation

Amoebae were grown on agar plates in association with *E. coli*, washed free of bacteria, and deposited (5×10^7 cells/filter) on Millipore filters to initiate development (0h). Cells were washed off the filters at various times with water and sonicated. The specific activity of α -D-galactosidase in the resulting homogenates was determined in control cells (●), cells treated with actinomycin D (100 μ g/ml) at 0h (○), cells treated with cycloheximide (0.5 mg/ml) at 0h (▲), and cells treated with cycloheximide (0.5 mg/ml) at 1.5h (Δ). Each point represents the mean of duplicate determinations. Specific activity is expressed as nmol of substrate hydrolysed/min per mg of protein under standard assay conditions. For further details, see the Materials and Methods section.

UDP-galactose must be required for the synthesis of the spore mucopolysaccharide and possibly for the synthesis of the discoidin receptor. A β -D-galactosidase had already been reported (Every & Ashworth, 1973) and we were able to detect an α -D-galactosidase with different properties and a separate developmental regulation from that of the β -D-galactosidase.

α -D-Galactosidase was found to be similar to other known exoglycosidases from *D. discoideum* in several respects. It binds to concanavalin A-Sepharose and is eluted with α -methyl glucoside, which might indicate that it is a glycoprotein, although we have no direct evidence for the presence of covalently attached sugar residues. It is likely therefore that the molecular weight estimated by gel filtration is not its true molecular weight (Andrews, 1964). It is an acidic protein, as shown by electrophoresis in starch gel and its behaviour on DEAE-cellulose. It displays optimal activity towards 4-methylumbelliferyl α -D-galactoside at acid pH, but negligible activity at neutral or alkaline pH.

The subcellular distribution of α -D-galactosidase, however, is not typical of the *D. discoideum* glyco-

Fig. 4. Effect of combined inhibitors of RNA synthesis on α -D-galactosidase accumulation

Amoebae were grown on agar plates in association with *E. coli*, washed free of bacteria, and deposited (5×10^7 cells/filter) on Millipore filters to initiate development (0h). At intervals, cells were washed off the filters and sonicated. The specific activity of α -D-galactosidase was then determined in control cells (●) and in cells treated with actinomycin D (100 μ g/ml) plus daunomycin (250 μ g/ml) at 0h (□). Each point represents the mean of duplicate determinations. Specific activity is expressed as nmol of substrate hydrolysed/min per mg of protein under standard assay conditions. For further details, see the Materials and Methods section.

sidases in general. About 80% of the α -D-galactosidase activity remaining after the removal of unbroken cells was soluble, and less than 20% was recovered in the lysosomal pellet. By comparison, about 70% of several other acid hydrolases, including β -N-acetyl-D-glucosaminidase and α -D-mannosidase, are localized in the lysosomal pellet prepared by the same method (Wiener & Ashworth, 1970; Ashworth & Quance, 1972). The *D. discoideum* α -D-galactosidase, however, is typical of α -D-galactosidases generally, since the enzyme from most organisms is present in the soluble fraction (Dey & Pridham, 1972).

Wiener & Ashworth (1970) have suggested that the lysosomal population is heterogeneous, and this would account for the independent regulation of several lysosomal enzymes and their secretion (Ashworth & Quance, 1972; Every & Ashworth, 1975). It has also been found that the integrity of *Dictyostelium* lysosomes depends on the conditions of homogenization (Wiener & Ashworth, 1970). It is therefore conceivable that some (if not most) of the α -D-galactosidase activity is located in lysosomal particles that are hyper-fragile and distinct from morphologically similar classical lysosomes.

α -D-Galactosidase, like several other glycosidases (Loomis, 1975), undergoes changes in specific activity during development. It is, however, unique among glycosidases so far studied in reaching its maximum specific activity during interphase by a process apparently requiring protein synthesis *de novo*. The significance of this increase is unclear, but may reflect a requirement for galactose to promote the synthesis of galacto-conjugates needed during early development, of which the discoidin receptor (Reitherman *et al.*, 1975) may be one. β -D-Galactosidase shows quite different kinetics of accumulation, rising very slightly during most of development, then doubling during fruiting-body formation. Since the highest specific activity of β -D-galactosidase occurs in mature fruits, it is possible that one of its functions is to degrade a spore-wall component during germination.

The intracellular increase in α -D-galactosidase specific activity during interphase could in principle arise by activation of a pre-existing enzyme, by an increase in enzyme synthesis relative to protein synthesis, or by a decrease in enzyme degradation relative to protein degradation. The use of inhibitors of protein synthesis helps to distinguish between the activation of an inactive form and the two possible mechanisms requiring protein synthesis *de novo*. Cycloheximide added to the lower-pad solution totally blocked the specific-activity increase of α -D-galactosidase, indicating that the increase is at least partly due to synthesis of the enzyme *de novo*. The specific activity of α -D-galactosidase fell slightly during interphase when amoebae were treated with cycloheximide; it is therefore likely that the rate of degradation of the enzyme is slightly greater than that of cellular protein generally.

Amoebae allowed to develop in the presence of actinomycin D accumulated α -D-galactosidase normally. Since actinomycin D has been shown to inhibit *D. discoideum* RNA synthesis by 95% in 15 min (Sussman *et al.*, 1967), this result might be an indication of a moderately stable mRNA species. However, Firtel *et al.* (1973) have shown that, although actinomycin D fully inhibits the synthesis of rRNA, mRNA synthesis is only inhibited by 70% in the presence of the drug. Moreover, they found that daunomycin, although only partially effective by itself, would in combination with actinomycin D inhibit mRNA synthesis almost completely. There have been several previous reports of stable mRNA species (Roth *et al.*, 1968; Loomis, 1969b), which implied translational control of enzyme synthesis, based on experiments with actinomycin D. Our finding that α -D-galactosidase accumulation was unaffected by actinomycin D alone even after 5 h, but was halted after 1 h when actinomycin D was used with daunomycin, provides independent confirmation of the work of Firtel *et al.* (1973) and suggests that the mRNA for β -galactosidase is relatively short-

lived. It is clearly of importance to interpret experiments using actinomycin D with caution, particularly in connexion with organisms whose DNA has a low (C+G) content.

α -D-Galactosidase was excreted during development and was detected in the lower-pad solution. The kinetics of intracellular and extracellular accumulation were such that the intracellular/extracellular specific-activity ratio was high during interphase, but fell to a correspondingly low ratio after aggregation. Ashworth & Quance (1972), after observing that the syntheses of several glycosidases were regulated by the nature of the organism's food source during growth and not by a developmental programme, suggested that the excretion of a glycosidase might be the developmentally significant event. Such circumstantial evidence as there is, however, argues against an extracellular role for α -D-galactosidase. α -D-Galactosidase has virtually no activity (towards 4-methylumbelliferyl α -D-galactoside) at pH 7.5, yet amoebae will develop on filters resting on pads saturated with buffered salts solution adjusted to a pH of 7.5 with NaOH at the same rate as they develop with the standard lower-pad solution (pH 6.1). When either galactose or galactono- γ -lactone was added to the lower-pad solution at a concentration several times the K_i value for the α -D-galactosidase-inhibitor interaction, little effect on development was observed. There is one possible explanation for glycosidase excretion that does not involve an extracellular enzymic function. Hickman & Neufeld (1972) have argued that, in cultured fibroblasts, lysosomal enzymes are primarily secreted and afterwards enter lysosomes by pinocytosis. It is possible that α -D-galactosidase and other *D. discoideum* glycosidases are also excreted in order to re-enter the cell, and specificity of uptake could be a mechanism for bringing about lysosomal heterogeneity.

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