Rates of Degradation and Synthesis of Glycosidases *de novo* during Growth and Differentiation of *Dictyostelium discoideum*

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1. Injection of a purified preparation of β -N-acetylglucosaminidase from the spent growth medium of myxamoebae of *Dictyostelium discoideum* into rabbits gave rise to an antibody preparation containing both anti- α -glucosidase and anti- β -acetylglucosaminidase activities. 2. These two activities were shown to reside in different immunoglobulin molecules and it was concluded that the β -N-acetylglucosaminidase preparation contained trace amounts of highly antigenic α -glucosidase. 3. A single precipitin band having β -N-acetylglucosaminidase activity was formed in Ouchterlony plates when this antibody preparation was tested against extracts obtained from differentiated cells or from myxamoebae grown either axenically or on bacteria. 4. The antibody preparation was used to show that both β -N-acetylglucosaminidase and α -glucosidase molecules are synthesized *de novo* from isotopically labelled amino acids during both the growth and differentiation phases of the life cycle and to show that neither of these proteins is significantly degraded during the growth phase or during the first 9 h of differentiation. 5. The rates of accumulation of these assayable enzyme activities are thus equal to their rates of synthesis during growth and early differentiation. 6. The factors regulating cellular enzyme activity during the life cycle of *D. discoideum* are discussed.

It has been suggested by Loomis and co-workers (Loomis, 1969, 1970*a,b*; Coston & Loomis, 1969) that the synthesis of the glycosidases N-acetylglucosaminidase, α -mannosidase and β -glucosidase during differentiation of *Dictyostelium discoideum* NC-4 is genetically regulated by the developmental programme. That is to say, at specific times during development certain genes are activated or inactivated, resulting in the initiation or termination of synthesis of these enzymes. Such enzymes have been termed 'developmentally controlled enzymes'.

Until recently, the three main criteria for classifying a slime-mould enzyme as developmentally controlled have been: (1) the specific activity of the enzyme increased more than threefold at a specific time during development; (2) the increase in specific activity was prevented by inhibitors of RNA and protein synthesis (such as actinomycin D and cycloheximide) and (3) morphologically and temporally deranged mutants were aberrant in their pattern of enzyme accumulation (Sussman & Sussman, 1969; Loomis, 1970*a,b*; Quance & Ashworth, 1972).

However, Wright and her co-workers (Pannbacker & Wright, 1966; Wright, 1968; Gustafson & Wright, 1972) maintain that these criteria are not sufficient for

the definition of a developmentally controlled enzyme. They pointed out that, besides gene activation, an increase in the specific activity of an enzyme could come about by epigenetic processes such as: (1) the activation of re-existing enzymes by diffusible activators; (2) the removal of diffusible inhibitors of enzyme activity; (3) the removal of non-diffusible 'masking' substances bound to enzyme; (4) the conversion of inactive precursor enzyme segments; and (5) a decrease of enzyme degradation against a background of steady enzyme synthesis. These workers suggest that inhibitors of RNA and protein synthesis, and morphogenetic mutations, could affect the accumulation of enzyme activity, by interfering with epigenetic control of the type described above rather than by interfering with specific enzyme synthesis as such.

Therefore it is imperative for an understanding of the regulation of 'developmentally controlled enzymes' that a method be found for determining the absolute rate of enzyme synthesis. In this paper we approached this problem by a method similar to that used by Schimke (1969) for determining the rates of enzyme synthesis in mammalian tissues. Thus an attempt was made to determine directly whether the accumulation of enzyme activity was due to synthesis *de novo*. Then the actual rate of accumulation of total assayable enzyme activity per cell (k_a) and the degradative rate constant for enzyme molecules (k_d) were determined. So, provided that the

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accumulation of enzyme activity results from protein synthesis *de novo*, the k_a and k_d are known, then the rate of enzyme synthesis per cell (k_s) can be calculated.

Materials and Methods

Materials

Complete Freund adjuvant was obtained from Difco Laboratories, Detroit, Mich., U.S.A. Rabbits were not inbred strains, but were those used as a routine at the Max-Planck-Gesellschaft, Tübingen, West Germany. Purified glycosidases were prepared by Every & Ashworth (1973). Human liver β -N-acetylglucosaminidase, prepared by $(\text{NH}_4)_2\text{SO}_4$ fractionation, was a gift from M. Carroll, Queen Elizabeth College, London, U.K. U- ^{14}C -labelled amino acid mixture (code CFB 104) of specific radioactivity 57 mCi/mg-atom of carbon and U- ^3H -labelled amino acid mixture (code TRA 263) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. *p*-Nitrophenyl glycosides and naphthol-AS-B1 acetamido-2-deoxy- β -D-glycopyranoside were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. All other chemicals were of the highest purity commercially available and were purchased either from BDH Chemicals Ltd., Poole, Dorset, U.K., or from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Methods

The methods used to grow and develop cells of *D. discoideum* Ax-2, prepare crude enzyme extracts and assay enzyme activity were the same as those described in the preceding paper (Every & Ashworth, 1975).

Preparation of anti-enzyme antibodies. A number of immunization programmes using complete Freund adjuvant and $\text{Al}(\text{OH})_3$ adjuvant were tried. The most successful preparation, and the one subsequently used for the experiments in this paper, was obtained by a slight modification of the method used by Beug *et al.* (1970). A solution containing purified β -N-acetylglucosaminidase and 2.5% (w/v) $\text{KAl}(\text{SO}_4)_2$ was adjusted to pH 6.5 with 0.1 M-KOH by using Bromocresol Purple as an indicator. The precipitate was spun down, resuspended in 0.9% NaCl, and injected into rabbits according to the following immunization schedule. Rabbits were injected intravenously with 1 ml on days 1, 3 and 5, subcutaneously throughout the body with 10×0.1 ml on days 1, 5 and 9, subcutaneously between the toes with 10×0.1 ml on days 3, 7 and 11 and intraperitoneally with 1 ml on days 1, 3, 5, 7, 9 and 11. To lessen the risk of anaphylactic shock in

the latter stages of the immunization, β -N-acetylglucosaminidase was dissolved in 0.9% NaCl without $\text{Al}(\text{OH})_3$ adjuvant for injection as follows: intravenously with 1 ml on days 7, 9, 11, 14, 16, 18 and 88, subcutaneously throughout the body with 10×0.1 ml on days 14, 18 and 88, subcutaneously between the toes with 10×0.1 ml on days 16 and 88, and intraperitoneally with 1 ml on days 14, 16, 18 and 88. In the first 18 days 1.8 mg of enzyme was injected and on day 88 1 mg of enzyme was injected.

Fresh blood samples were collected 1 week after the last injection. After the blood had been allowed to clot at 23°C for 2 h followed by 16 h at 4°C, the serum was collected by centrifugation and stored at -20°C. IgG (immunoglobulin G) was prepared from this serum by fractionation in 43%-satd. $(\text{NH}_4)_2\text{SO}_4$ and chromatography on DEAE-cellulose by the method of Levy & Sober (1960).

Serological reactions. The Ouchterlony double-diffusion precipitation reaction was carried out essentially as described by Ouchterlony (1958), by using 1% agar in 0.075 M-sodium phosphate buffer, pH 7.1, containing 0.43% NaCl. The activity of β -N-acetylglucosaminidase was detected on the surface of the double-diffusion agar plates by histochemical enzyme-staining techniques by using the substrate naphthol-AS-B1 acetamido-2-deoxy- β -D-glucopyranoside as described by Hayashi (1965) and Raunio (1969).

The microquantitative precipitin reaction was performed essentially as described by Kabat & Meyer (1961) and Campbell *et al.* (1963). Antigen samples and IgG solution, which had been centrifuged at 30000g for 60 min to remove any debris, were mixed with barbital buffer (pH 6.9, 1.02; Kabat & Meyer, 1961) to a total volume of 0.2 ml in chromic acid-cleaned Dreyer's agglutination tubes (60 mm $\times$ 7 mm). The mixtures were kept at room temperature (18–22°C) for 2 h and then at 4°C for 2–3 days. The precipitate was collected at 4°C, washed three times with barbital buffer (0.2 ml) and assayed for protein by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The supernatants were assayed for glycosidase activity.

Measurement of isotopically labelled enzymes. Labelled amino acids were incorporated into β -N-acetylglucosaminidase and α -glucosidase during growth, by adding either 50 μCi of a U- ^{14}C -labelled amino acid mixture (see under 'Materials') or 100 μCi of a mixture of amino acids labelled with ^3H (see under 'Materials') to 70 ml cultures of axenically growing myxamoebae of strain Ax-2 (5×10^4 cells/ml). The cells were harvested at a cell density of 3×10^6 cells/ml.

During differentiation labelled amino acids were incorporated into the enzymes as follows. Myxamoebae grown axenically to 3×10^6 cells/ml were

EXPLANATION OF PLATE I

Ouchterlony double-diffusion precipitin analysis of anti-enzyme antibody versus β -N-acetylglucosaminidase and α -glucosidase from various sources

For full experimental details see under 'Methods'. (a) and (c) show the protein precipitation bands of the antibody-enzyme reactions. (b) shows the staining reaction of the β -N-acetylglucosaminidase-antibody precipitation bands of (a) with naphthol-As B1-glucosaminide substrate. (d) shows a sketch of the protein precipitation bands of (c). The central well contains 10 μ l of the following enzyme antigen preparations: N1, purified β -N-acetylglucosaminidase (0.08 mg/ml); N, β -N-acetylglucosaminidase partially purified by Sephadex G-200 chromatography as described by Every & Ashworth (1973) (1.5 mg/ml); DB, crude enzyme extract from 8 h-differentiated cells which had been grown in bacterial suspension; GB, crude enzyme extract from cells grown exponentially in bacterial suspension; DA, crude enzyme extract from cells grown axenically; α G, purified α -glucosidase (2.5 mg/ml).

washed and suspended at 10^7 cells/ml in 20 ml of 0.0167 M-sodium phosphate buffer, pH 6.0, containing 50 μ Ci of a U- 14 C-labelled amino acid mixture and shaken for 9 h at 22°C.

The isolation of labelled enzymes for measurement of radioactivity was performed by a combination of immunoprecipitation and polyacrylamide-gel-electrophoresis techniques essentially as described by Franke & Sussman (1971). The cell sample was washed twice with ice-cold water, sonicated (in an MSE, 100 W disintegrator with peak-to-peak amplitude of 9 μ m for four 15 s periods), centrifuged at 30000g for 1 h and the supernatant assayed for enzyme activities. Anti-enzyme antibody was added to the cell extract in excess of the equivalence point and the mixture was incubated at room temperature for 2 h, then at 4°C for 3 days. The precipitate was washed twice with barbital buffer by centrifugation, heated in a boiling-water bath with sodium dodecyl sulphate and mercaptoethanol, and then subjected to sodium dodecyl sulphate-polyacrylamide-gel electrophoresis as described by Every & Ashworth (1973). The exact location of the enzyme polypeptide chains in the gel was determined by comparison of the Coomassie Blue-stained gel with gels calibrated with standard proteins of known molecular weight and purified β -N-acetylglucosaminidase and α -glucosidase as described by Every & Ashworth (1973). The gel was then cut into 2 mm slices, placed into glass scintillation vials (two slices/vial), dried for 1 h at 55°C, and then dissolved in 0.5 ml of H₂O₂-conc. NH₃ (sp.gr. 0.88) (99:1, v/v) at 55°C overnight (Goodman & Matzura, 1971). Then 5 ml of Triton-toluene scintillation fluid, containing 700 ml of toluene, 300 ml of Triton X-100 and 5 g of 2,5-diphenyloxazole, was added to each vial and radioactivity determined by the method of Fox (1968) in a Packard model 432 Tri-Carb liquid-scintillation spectrometer. This procedure was repeated without the addition of labelled amino acids and the apparent radioactivity of these control gels was subtracted from the test gels.

Determination of rate of enzyme degradation during growth. α -N-Acetylglucosaminidase and α -glucosidase were labelled with 14 C-labelled amino acids during axenic growth of myxamoebae as described above. The cells were washed twice with fresh non-radioactive axenic medium by centrifugation and resuspended to 3×10^6 cells/ml in fresh axenic medium (81 ml) containing a 15 mM mixture of unlabelled amino acids of the same composition as the original 14 C-labelled amino acid mixture. The 250 ml Erlenmeyer flask containing the cell suspension was shaken at 150–170 rev./min so that growth could continue. All manipulations were performed under sterile conditions. Samples (20 ml) were removed at 3 h intervals and frozen. The total cellular and

extracellular radioactivity present in β -N-acetylglucosaminidase and α -glucosidase was then determined by the radioimmunological technique described in the previous section.

Determination of the rate of enzyme degradation during differentiation. β -N-Acetylglucosaminidase and α -glucosidase were labelled with 3 H-labelled amino acids during axenic growth of myxamoebae, as described above. The cells were washed three times with 0.0167 M-sodium phosphate buffer, pH 6.0, and resuspended to 10^7 cells/ml in 25 ml of phosphate buffer containing a 15 mM mixture of unlabelled amino acids of the same composition as the 3 H-labelled amino acid mixture used for labelling the enzymes. The 50 ml Erlenmeyer flask containing the cell suspension was shaken at 150–170 rev./min to allow differentiation to proceed. Under these conditions amino acids are taken up passively by the amoebae and do not inhibit the development of aggregation competence (Lee, 1972). Samples (6 ml) were frozen at 3 h intervals and later estimated for above total cellular and extracellular radioactivity present in β -N-acetylglucosaminidase and α -glucosidase as described in a previous section.

Results

Characterization of the precipitin reaction

The anti-enzyme-precipitating activity of the serum was almost entirely recovered in the purified IgG fraction and this fraction was used in all subsequent experiments.

Ouchterlony tests were used to give an estimate of the strength and specificity of the anti-enzyme antibody preparations. Plate 1(a) shows that when equal amounts of β -N-acetylglucosaminidase from different sources (measured by catalytic activity) were tested with the equivalent amount of anti-enzyme antibody, a single precipitin band formed. The complete fusion of the bands at all intersections indicates that β -N-acetylglucosaminidase preparations from these sources are antigenically identical. These bands had strong β -N-acetylglucosaminidase activity when stained histochemically (Plate 1b).

With crude β -N-acetylglucosaminidase extracts prepared from axenically grown cells a second weaker precipitation band appeared on the Ouchterlony tests, which seems to correspond to a separate α -glucosidase-antibody precipitation system (Plates 1b and 1d).

Characterization of the quantitative precipitin reaction is shown in Fig. 1, which demonstrates that the equivalence point (arrow A) for the β -N-acetylglucosaminidase-antibody precipitin reaction can be precisely established by assaying the supernatant fraction for the appearance of unprecipitated enzyme

Fig. 1. Quantitative precipitin analysis of the β -N-acetylglucosaminidase-anti-enzyme antibody reaction

A constant amount of anti-enzyme antibody (0.1 mg of IgG) was mixed with increasing amounts of purified β -N-acetylglucosaminidase as indicated on the abscissa (the purified enzyme was mixed with 1 mg of bovine serum albumin/ml). As a control, different quantities of enzyme were mixed with a constant amount of normal rabbit IgG (0.1 mg). After incubation of the mixtures and separation of any precipitates from the supernatants, measurements were made of total protein in the antibody-enzyme precipitate ($\bullet$), total enzyme activity in the supernatant ($\circ$), and total enzyme activity in the supernatants of the control reactions of enzymes and normal IgG ($\square$). For full experimental details see under 'Methods' and for an explanation of the arrows A and B and the dotted line see the Results section. The results of two experiments are shown.

activity. At the equivalence point, the amount of antibody required to precipitate enzyme activity was directly proportional to enzyme concentration over a 100-fold range of dilutions; the ratio of catalytic to antigenic activity at equivalence (units of enzyme precipitated/ μ g of IgG added) being $1:68.9 \pm 1.5$. Results are given as mean $\pm$ S.E.M. for 10 experiments.

The antibody-enzyme complex was not solubilized in the region of antibody excess, but still retained approx. 20–35% of the original β -N-acetylglucosaminidase activity. However, in the region of antigen excess, 42% of the antibody-enzyme complex became solubilized. If this solubilized complex was totally inactive, then one would expect the appearance of unprecipitated enzyme activity after the equivalence point to produce the graph shown by the dotted line in Fig. 1. On the other hand, if the 42% solubilized antibody-enzyme complex retained enzyme activity, then at the point indicated by arrow B in Fig. 1, the complex would be expected to contribute 0.59 unit of enzyme more to supernatant enzyme activity than shown by the dotted line at this

point. This must be the case, because the actual difference in enzyme activity between the hypothetical dotted graph and the experimental graph at point B is 0.58 unit. Thus the solubilized antibody β -N-acetylglucosaminidase complex, in the region of antigen excess, retained full enzyme activity.

Immunotitration experiments

Fig. 2(a) shows the effect of adding increasing amounts of β -N-acetylglucosaminidase (measured in activity units) to a constant amount of antibody, and Fig. 2(b) shows the converse experiment. The equivalence points for crude β -N-acetylglucosaminidase preparations from exponentially grown myxamoebae and differentiated slime-mould cells were identical (within experimental error) with the equivalence point for purified slime-mould enzyme. This indicates that active β -N-acetylglucosaminidase molecules from these sources are antigenically identical.

The anti-N-acetylglucosaminidase antibody preparation shows no cross-reactivity with α -mannosidase, β -glucosidase or human liver β -N-acetylglucosaminidase (Figs. 2b and 3). On the other hand, the antibody preparation did precipitate α -glucosidase activity. However, with samples containing different ratios of α -glucosidase to β -N-acetylglucosaminidase activities there were no effects on the ratio of β -N-acetylglucosaminidase catalytic to antigenic activity at the equivalence point. Thus α -glucosidase does not cross-react with the antibody that binds to β -N-acetylglucosaminidase, i.e. α -glucosidase is precipitated by a different antibody from the antibody that precipitates β -N-acetylglucosaminidase. This conclusion agrees with the observations made on the Ouchterlony tests discussed previously (Plates 1c and 1d). Thus, although the antibody preparation was prepared primarily for studies on β -N-acetylglucosaminidase, it has also proved suitable as a tool for the isolation of radioactively labelled α -glucosidase by immunological techniques.

Enzyme synthesis de novo

Fig. 4 shows the distribution of radioactivity in sodium dodecyl sulphate-polyacrylamide gels after electrophoresis of labelled enzymes, which were isolated by immunoprecipitation procedures from 'growth' and 'differentiation' cell samples. Although a considerable amount of extraneous radioactivity was co-precipitated with the enzymes during the immunoprecipitation procedure, it was satisfactorily separated from the enzymes by the sodium dodecyl sulphate-polyacrylamide-gel-electrophoresis technique. Thus it is most probable that the two peaks of radioactivity in the gel, which coincide with the

Fig. 2. Immunotitration of β -N-acetylglucosaminidase from various sources with anti-enzyme antibody

(a) A constant amount of anti- β -acetylglucosaminidase antibody (43 μ g of IgG/ml) was mixed with increasing amounts (as indicated on the abscissa) of β -N-acetylglucosaminidase from the following sources: $\circ$, purified *D. discoideum* enzyme (mixed with 1 mg of bovine serum albumin/ml); Δ , crude enzyme extract from vegetative amoebae grown on *E. coli* B/r; $\blacktriangle$, crude enzyme extract from 10h-differentiated cells initially grown on *E. coli* B/r; $\square$, crude enzyme extract from vegetative amoebae grown axenically; $\blacksquare$, crude enzyme extract from 10h-differentiated cells initially grown axenically; $\bullet$, human liver β -N-acetylglucosaminidase extract. After incubation of the mixture and removal of any precipitate the enzyme activities remaining in the supernatant were determined. For full experimental details see under 'Methods'. Control mixtures containing normal rabbit IgG and the various β -N-acetylglucosaminidase preparations had no precipitation of enzyme activity. (b) Constant amounts of β -N-acetylglucosaminidase (1.13 units/ml) from various sources were mixed with increasing amounts of antibody as indicated on the abscissa. The sources of enzyme and the symbols are the same as in (a). After incubation of the various mixtures and removal of any precipitate the enzyme activities remaining in the supernatants were determined. For full experimental details see under 'Methods'. Control mixtures containing normal rabbit IgG and the various enzyme preparations had no precipitation of enzyme activity.

position of β -N-acetylglucosaminidase and α -glucosidase polypeptide chains, represent incorporation of labelled amino acid into these two enzymes. These results therefore indicate that the increases in assayable enzyme in growth cultures and differentiating cells results, at least in part, from enzyme synthesis *de novo*.

Rates of enzyme degradation and synthesis

Although active enzyme molecules may accumulate by protein synthesis *de novo*, it is possible that the number of these molecules will be a net effect of continual synthesis and degradation of enzyme protein. A simple formulation for expressing changes in enzyme amount, which considers both synthesis and degradation, has been developed in several laboratories (Berlin & Schimke, 1965; Price *et al.*, 1962; Segal & Kim, 1963). Thus a change in enzyme amount is expressed by:

$$\frac{dE}{dt} = k_s - k_d E \quad (1)$$

where E is the total enzyme units per unit cell mass at time t , k_s is a zero-order rate constant of enzyme

synthesis *de novo*, expressed as enzyme units $\cdot$ time $^{-1} \cdot$ mass $^{-1}$, and k_d is a first-order rate constant for enzyme degradation, expressed as time $^{-1}$.

If units of cell number are used instead of cell mass then the term on the left-hand side of eqn. (1) becomes equivalent to the accumulation rate constant, k_a , determined in the preceding paper (Every & Ashworth, 1975). Thus

$$k_a = k_s - k_d E \quad (2)$$

where k_a is expressed as enzyme units $\cdot$ time $^{-1} \cdot$ cell number $^{-1}$, E is the total enzyme units per unit cell number, and k_s is a synthesis rate constant expressed as enzyme units $\cdot$ time $^{-1} \cdot$ cell number $^{-1}$.

Since k_a has been determined for the glucosidases in the preceding paper (Every & Ashworth, 1975), we can calculate k_s from eqn. (2) if k_d is estimated experimentally. Fig. 5 indicates that there is essentially no degradation of β -N-acetylglucosaminidase and α -glucosidase during the first 9 h of development, whereas the rate of degradation of total protein during the first 3 h of development is approx. 5%/h. G. Weeks & J. M. Ashworth (unpublished work) found a degradation rate of total protein of approx. 5%/h at the 3–4 h development stage of *D. discoideum*

Fig. 3. Immunotitration of various *D. discoideum* glycosidases with antibody produced against β -N-acetylglucosidase

A constant amount of crude cell extract from 10h-differentiated slime-mould cells which were grown axenically was mixed with increasing amounts of antibody as indicated on the abscissa. After incubation of the mixtures and removal of any precipitate the supernatants were assayed for various glycosidase activities. For full experimental details see under 'Methods': 10 arbitrary enzyme units of activity (1 unit = $\mu\text{mol}/\text{min}$) are equivalent to 1 unit/ml for β -N-acetylglucosaminidase (○), 0.04 unit/ml for α -mannosidase (■), 0.02 unit/ml for α -glucosidase (▲) and 0.005 unit/ml for β -glucosidase (□).

Ax-2, and Wright & Anderson (1960) found a protein turnover rate of 7%/h during culmination of *D. discoideum* NC-4.

When the accumulation of β -N-acetylglucosaminidase activity at the 4h development stage is prevented by adding cycloheximide (Loomis, 1969) enzyme activity remains relatively stable for many hours, whereas total protein is degraded at the rate of approx. 8%/h (G. Weeks & J. M. Ashworth, unpublished work). This also suggests that the degradation rate of β -N-acetylglucosaminidase is essentially zero.

In experiments to determine the rates of degradation of enzyme during growth, it was difficult to recover the immunoprecipitate from the large volumes of growth sample required, and the radioactivity peaks in the polyacrylamide gels, corresponding to β -N-acetylglucosaminidase and α -glucosidase samples, were rather low. However, the results indicated that there is essentially no degradation of enzyme. This conclusion is consistent with the essentially zero rate of degradation of total protein during exponential growth of myxamoebae of strain Ax-2 on axenic medium (G. Weeks & J. M. Ashworth, unpublished work).

Fig. 4. Incorporation of radioactive amino acids into β -N-acetylglucosaminidase and α -glucosidase during growth and differentiation of *D. discoideum* Ax-2

The distribution of radioactivity was measured in sodium dodecylsulphate-polyacrylamide gels after electrophoresis of enzyme samples extracted by immunological procedures from myxamoebae grown axenically (○) and from cells after 9h or development (●) in the presence of labelled amino acids. The density of the protein stain in the gels was measured by microdensitometry by using light of wavelength 595nm. The arrows mark the positions of (1) Bromophenol Blue, (2) L-chain of IgG, (3) H-chain of IgG, (4) β -N-acetylglucosaminidase and (5) α -glucosidase. For full experimental details see under 'Methods'.

Fig. 5. Rates of degradation of β -N-acetylglucosaminidase and α -glucosidase molecules during development of *D. discoideum* Ax-2

For full experimental details see under 'Methods'. One arbitrary unit of enzyme activity is equal to one unit of β -N-acetylglucosaminidase activity (●) or 0.01 unit of α -glucosidase activity (■). Radioactivity was determined in β -N-acetylglucosaminidase (○), α -glucosidase (□), and in total cellular plus extracellular protein (△).

Thus it is concluded that during exponential growth and differentiation k_d equals zero, and therefore

$$k_s = k_a$$

In the preceding paper (Every & Ashworth, 1975) it was shown that the rate of accumulation of β -N-acetylglucosaminidase and α -glucosidase remains essentially constant throughout exponential growth and the first few hours of differentiation, and therefore the rates of synthesis of these enzymes also do not change in going from exponential growth to the differentiation phase.

Absence of diffusible inhibitors and activators of glycosidase activity

When crude cell extracts of *D. discoideum* Ax-2, grown exponentially on bacteria, were mixed with extracts of cells taken at various stages of development (6, 12 and 24h samples), the assayable glycosidase activities were strictly additive. When the above crude cell extracts were incubated with purified glycosidases at 22°C for 30min, then assayed for glycosidase activity, the activities were also strictly additive. These results indicate that there are no diffusible inhibitors and activators of the glycosidases present in excess to account for the changes in cellular glycosidase amounts during growth and differentiation. This supports the conclusion that changes in the assayable enzyme activity result from enzyme synthesis *de novo*.

Discussion

It has been suggested that the period and rate of accumulation of β -N-acetylglucosaminidase is controlled genetically by the developmental programme

of the cellular slime mould, and that this may be an example of differential gene activation (Loomis, 1969, 1970b). In the present paper we have examined these suggestions by determining the actual rates of β -N-acetylglucosaminidase synthesis *de novo* to see if there is, in fact, any increase during development corresponding to specific gene activation.

First it must be shown that assayable enzyme activity accumulates as a result of enzyme synthesis *de novo*. The following evidence, when taken together, does indeed indicate that this is the case.

(a) Loomis (1969) found that actinomycin D inhibited the accumulation of assayable β -N-acetylglucosaminidase activity 2h after it was added to developing slime-mould cells. This has been interpreted as meaning that synthesis of the mRNA which is translated into enzyme molecules is required, and this mRNA is stable for about 2h. However, Gustafson & Wright (1972) point out that actinomycin D is known to have various non-specific detrimental effects on metabolism and they suggest that this drug may affect enzyme accumulation indirectly, by preventing underlying metabolic changes which are required for the inactivation of pre-existing inactive enzyme molecules.

(b) Cycloheximide stopped the accumulation of β -N-acetylglucosaminidase activity when added during the first few hours of development (Loomis, 1969). This has been interpreted as meaning that translation of enzyme molecules from mRNA is required for the increase in specific activity.

(c) The results in this paper and those of Loomis (1969) indicate that there are no diffusible activators or inhibitors of β -N-acetylglucosaminidase activity in excess during growth or differentiation. Thus it seems that the increase in specific activity of enzyme during aggregation cannot be explained by the removal of an inhibitor or the appearance of an activator.

(d) Wright and her co-workers (Killick & Wright, 1972) have shown that trehalose 6-phosphate synthetase activity in amoebae extracts is 'masked' by an inhibitor bound to the enzyme, which can be removed by $(\text{NH}_4)_2\text{SO}_4$ fractionation of the enzyme. They postulate that during differentiation metabolic changes cause the 'unmasking' of the enzyme and the specific activity increases. They suggest that actinomycin D may interfere with this process. No 'unmasking' of β -N-acetylglucosaminidase activity in growth-phase samples was detected after $(\text{NH}_4)_2\text{SO}_4$ fractionation or at any other stage during the purification of the enzyme (Every & Ashworth, 1973).

(e) The results in the present paper show that as assayable β -N-acetylglucosaminidase activity increases during development there is an exactly proportional increase in the amount of specific

anti-enzyme antibody required to precipitate enzyme activity. This indicates that active enzyme molecules are accumulating in exact proportion to the accumulation of assayable enzyme activity, but does not rule out the possibility that non-antigenic enzymically inactive precursor molecules are converted into antigenic enzymically active molecules.

(f) The most convincing evidence demonstrating synthesis of β -N-acetylglucosaminidase *de novo* is the incorporation of radioactively labelled amino acids into enzyme molecules as shown in this paper.

The results in this paper also indicate that α -glucosidase accumulates as a result of enzyme synthesis *de novo* for the same reasons cited for β -N-acetylglucosaminidase in (c), (d) and (f) above. However, it was also shown that the increase in the specific activities of these two enzymes at the beginning of development of the slime mould do not correspond to any change in the rate of enzyme synthesis. Therefore the genes coding for these two enzymes are not activated at the beginning of development.

Thus it seems that the genes are activated when cell growth is initiated, the enzymes are synthesized at a constant rate throughout exponential growth and the first few hours of differentiation, and the specific activity or cellular activity of the enzymes fluctuate according to changes in the rate of general protein synthesis and degradation or in the rate of cell division. After several hours of development enzyme synthesis ceases and the amount of enzyme in the cell becomes constant or declines.

It is interesting to note that once the accumulation of activity has stopped after growth has ceased, or after the development of aggregation competence, it cannot be re-initiated by disaggregating the cells and placing them on solid substratum so that normal development can proceed (Fig. 6; Newell *et al.*, 1972).

Thus it appears that β -N-acetylglucosaminidase synthesis can only be reinitiated by inoculating spores or the above disaggregated cells into fresh growth medium. In contrast, several other so-called 'developmentally controlled enzymes' [UDP-galactose epimerase, trehalose phosphate synthetase, UDP-glucose pyrophosphorylase and UDP-galactose transferase (Newell *et al.*, 1971, 1972)], which have ceased to accumulate at certain morphological stages of development, can be made to accumulate by disaggregating the cells and placing them in an environment allowing recapitulation of the various morphological stages. Thus although the re-induction of accumulation of these enzymes is correlated with distinct morphological stages, the re-induction of β -N-acetylglucosaminidase is not.

During this study on the regulation of glycosidase amounts it has become apparent that two basically

Fig. 6. Activity of β -N-acetylglucosaminidase in cells grown and developed under various conditions

Myxamoebae were grown in suspensions of *E. coli* B/r and either (a) washed and suspended in 0.0167 M-sodium phosphate buffer, pH 6, to develop aggregation competence, followed by disaggregation of the cells and placing them on solid substratum to develop into fruiting bodies, or (b) washed and placed on solid substratum to develop into fruiting bodies, or (c) allowed to enter the stationary phase of growth. Cell samples were collected for enzyme assays and protein determination. For full experimental details see under 'Methods'. $\circ$, Specific activity of N-acetylglucosaminidase; —, number of myxamoebae/ml of culture.

different mechanisms for modulation of specific protein concentrations occur at different stages of the cellular-slime-mould life cycle. During the exponential growth phase of *D. discoideum*, protein degradation occurs at a very low rate (G. Weeks & J. M. Ashworth, unpublished work), and in this respect it behaves like a typical exponentially growing microbe (Mandelstam, 1960). Thus, as for

growing bacteria (Jacob & Monod, 1961; Epstein & Beckwith, 1968), the regulation of enzyme amounts in *D. discoideum* must be dependent on the rate of specific enzyme synthesis relative to the rate of general protein synthesis. Proteins that are not needed are thus not synthesized and will be diluted out by cell growth and division, whereas proteins that are needed will be synthesized continuously. The regulation of β -N-acetylglucosaminidase concentrations during growth certainly seems to be of this type. During the development phase of *D. discoideum*, protein synthesis occurs against a background of considerable protein degradation (Gregg *et al.*, 1954; Wright & Anderson, 1960; White & Sussman, 1961; G. Weeks & J. M. Ashworth, unpublished work; Hames & Ashworth, 1974; Every & Ashworth, 1973) and in this respect it behaves like higher-plant and animal cells (Eagle, 1965; Pine, 1967; Filner *et al.*, 1969). In animal cells the rates of degradation of different proteins are regulated independently from each other and from the rates of synthesis, and it is clear that both degradation and synthesis of protein are important in the regulation of enzyme concentrations (Schimke, 1969; Schimke & Doyle, 1970). This also appears to be the case during the developmental phase of the cellular slime mould, as we have shown for the glycosidases.

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References

- Berlin, C. M. & Schimke, R. T. (1965) *Mol. Pharmacol.* **1**, 149–156
- Beug, H., Gerisch, G., Kempff, S., Riedel, V. & Cremer, G. (1970) *Exp. Cell Res.* **63**, 147–158
- Campbell, D. J., Garvey, J. S., Cremer, N. E. & Sussdorf, D. H. (1963) *Methods in Immunology*, W. A. Benjamin, New York and Amsterdam
- Coston, M. B. & Loomis, W. F. (1969) *J. Bacteriol.* **100**, 1208–1217
- Eagle, H. (1965) *Science* **148**, 42–51
- Epstein, W. & Beckwith, J. R. (1968) *Annu. Rev. Biochem.* **37**, 411–436
- Every, D. & Ashworth, J. M. (1973) *Biochem. J.* **133**, 37–47
- Every, D. & Ashworth, J. M. (1975) *Biochem. J.* **148**, 161–167.
- Filner, P., Wray, J. L. & Varner, J. E. (1969) *Science* **165**, 358–367
- Fox, B. W. (1968) *Lab. Pract.* **17**, 595–598
- Franke, J. & Sussman, M. (1971) *J. Biol. Chem.* **246**, 6381–6388
- Goodman, D. & Matzura, H. (1971) *Anal. Biochem.* **42**, 481–486
- Gregg, J. H., Hackney, A. L. & Krivanek, J. O. (1954) *Biol. Bull.* **107**, 226–235
- Gustafson, G. L. & Wright, B. E. (1972) *CRC Crit. Rev. Microbiol.* **1**, 453–478
- Hames, B. D. & Ashworth, J. M. (1974) *Biochem. J.* **142**, 301–315
- Hayashi, M. (1965) *J. Histochem. Cytochem.* **13**, 355–360
- Jacob, F. & Monod, J. (1961) *J. Mol. Biol.* **3**, 318–356
- Kabat, E. A. & Meyer, M. M. (eds.) (1961) *Experimental Immunochimistry*, 2nd edn., Thomas, Springfield, Ill.
- Killick, K. A. & Wright, B. E. (1972) *J. Biol. Chem.* **247**, 2967–2969
- Lee, K. C. (1972) *J. Gen. Microbiol.* **72**, 457–471
- Levy, H. B. & Sober, H. A. (1960) *Proc. Soc. Exp. Biol. Med.* **103**, 250–252
- Loomis, W. F. (1969) *J. Bacteriol.* **97**, 1149–1154
- Loomis, W. F. (1970a) *J. Bacteriol.* **103**, 375–381
- Loomis, W. F. (1970b) *Exp. Cell Res.* **60**, 285–291
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mandelstam, J. (1960) *Bacteriol. Rev.* **24**, 289–308
- Newell, P. C. (1971) *Essays Biochem.* **7**, 87–126
- Newell, P. C., Longlands, M. & Sussman, M. (1971) *J. Mol. Biol.* **58**, 541–544
- Newell, P. C., Franke, J. & Sussman, M. (1972) *J. Mol. Biol.* **63**, 373–382
- Ouchterlony, O. (1958) *Progr. Allergy* **5**, 1–78
- Pannbacker, R. G. & Wright, B. E. (1966) *Biochem. Biophys. Res. Commun.* **24**, 334–339
- Pine, M. J. (1967) *Cancer Res.* **27**, 522–525
- Price, V. T., Sterling, W. R., Tarantola, V. A., Hartley, R. W. & Recheigl, M., Jr. (1962) *J. Biol. Chem.* **237**, 3468–3475
- Quance, J. & Ashworth, J. M. (1972) *Biochem. J.* **126**, 609–615
- Raunio, V. (1969) *Acta Pathol. Microbiol. Scand. Suppl.* **195**, 1–48
- Schimke, R. T. (1969) *Curr. Top. Cell. Regul.* **1**, 77–124
- Schimke, R. T. & Doyle, D. (1970) *Annu. Rev. Biochem.* **39**, 929–976
- Segal, H. L. & Kim, Y. S. (1963) *Proc. Nat. Acad. Sci. U.S.A.* **50**, 912–918
- Sussman, M. & Sussman, R. R. (1969) *Symp. Soc. Gen. Microbiol.* **19**, 403–435
- White, G. J. & Sussman, M. (1961) *Biochim. Biophys. Acta* **53**, 285–293
- Wright, B. E. (1968) *J. Cell. Physiol.* **72**, Suppl. **1**, 145–160
- Wright, B. E. & Anderson, M. L. (1960) *Biochim. Biophys. Acta* **43**, 62–66