Enzyme Synthesis in the Cellular Slime Mould *Dictyostelium discoideum* during the Differentiation of Myxamoebae Grown Axenically

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1. Myxamoebae of the cellular slime mould *Dictyostelium discoideum* Ax-2 were grown on different media, and were harvested either in the stationary or exponential phases of the growth cycle to yield samples of myxamoebae differing in enzymic composition. 2. Morphogenesis and cell differentiation phenomena in *D. discoideum* appear to be similar in myxamoebae grown and harvested under different conditions. 3. The specific activity of the enzymes β -N-acetylglucosaminidase, acid phosphatase, α -mannosidase, β -glucosidase and alkaline phosphatase have been determined during cell differentiation of myxamoebae grown and harvested under different conditions. 4. The pattern of synthesis of these enzymes, all of which have been claimed to be part of the 'developmental programme', either remains unaffected despite the origin of the myxamoebae (alkaline phosphatase) or is qualitatively similar but quantitatively affected (acid phosphatase, β -glucosidase) or is both qualitatively and quantitatively affected by changes in the myxamoebae (α -mannosidase, β -N-acetylglucosaminidase). 5. The implications of these results for the concept of a 'developmental programme' are discussed.

There is evidence that, during cell differentiation phenomena in general, and the differentiation of *Dictyostelium discoideum* myxamoebae in particular, some enzymes are synthesized preferentially (Sussman & Sussman, 1969). This has given rise to the suggestion that the 'developmental programme' that controls all differentiation phenomena merely specifies the time and the extent of synthesis of particular enzymes. As it is believed that protein synthesis is an essentially similar process in all organisms and as the Jacob & Monod (1961) operon theory has proved to be basically correct in describing how protein synthesis is controlled in *Escherichia coli* it has been suggested that most cell differentiation phenomena can be explained by differential gene activity; the 'developmental programme' therefore specifies the order in which genes become active and the amount of products that they produce. There is genetic evidence for differential gene activity in *D. discoideum* (Loomis, 1969b) and many of the observations on the effect of inhibitors of protein synthesis on the appearance of enzyme activities during cell differentiation (Roth *et al.*, 1968) can be accounted for by the theory that controls exist at the levels of transcription and translation of the appropriate gene(s). However, although synthesis of an enzyme might be causally related to a cell differentiation event and although such changes might be a necessary part of any explanation of cell differentiation it is not clear that the mere description of such events provides a sufficient explanation for cell

differentiation. It is, however, difficult to devise experiments to test this assertion.

The observation that the chemical and enzymic composition of myxamoebae of *D. discoideum* Ax-2 can be altered by changing the growth medium (Watts & Ashworth, 1970; Ashworth & Quance, 1972) and that, despite such changes, the myxamoebae will still differentiate (Ashworth & Watts, 1970), allows the above assertion to be tested. Thus it is possible to study the biochemical events that occur in two different cell populations that are going through the same morphogenetic sequence but that have, initially, different chemical and enzymic compositions. If it is both a necessary and sufficient explanation of the formation of a morphogenetic stage in the differentiation process that the stage contains a specified assembly of enzymic activities at certain specified concentrations then, despite their initially different compositions, both cell populations should, by the time that that stage is reached, have attained a similar assembly of those enzymes believed to form part of the 'developmental programme'. If, however, the two cell populations preserve their distinguishing enzymic characteristics and yet form the same morphogenetic stage then the implication is either that the synthesis of those enzymes was not part of the 'developmental programme' or that the 'developmental programme' specifies events in addition to the order in which genes become active and the amount of products that they produce. In the present paper we describe experiments that support these implications.

Materials and Methods

The chemicals used, enzyme assays and methods used to grow the myxamoebae of *D. discoideum* Ax-2 were the same as those described in the preceding paper (Ashworth & Quance, 1972).

Preparation of samples of cells undergoing differentiation

In the present paper we describe some biochemical consequences of allowing myxamoebae, grown under different conditions, to differentiate. Bacterially grown cells were grown on *Aerobacter aerogenes* as nutrient on the surface of Petri dishes as described by Sussman (1966). The myxamoebae were harvested and washed with cold water when the plates were virtually free of bacteria but before there were any visible signs of aggregation. The washed myxamoebae were deposited on well-washed Millipore filter supports (0.8 μ m pore size, 47mm diam., Cat. no. AABP 04700) supported on pads containing KCl, $MgCl_2$ and 50mm-sodium-potassium phosphate buffer, pH 6.5, as described by Sussman (1966) but at

cell densities of 5×10^7 myxamoebae/filter as well as the more usual 10^8 myxamoebae/filter. These filters were incubated at 22–23°C and cells were harvested with 3 ml of water and the suspensions stored frozen. Two kinds of axenic medium were used to grow the myxamoebae as described by Watts & Ashworth (1970). One contained peptone, yeast extract and phosphate buffer, pH 6.7 (axenic medium), and the other contained, in addition, 86mm-D-glucose (axenic medium+glucose). Myxamoebae were harvested either when in the exponential or the stationary phase of growth. Half of a 700ml culture of myxamoebae in the exponential phase (2×10^6 – 3×10^6 myxamoebae/ml of medium) was harvested and the myxamoebae were washed and resuspended in cold water and distributed on Millipore filter supports as described above at a cell density of 5×10^7 myxamoebae/filter. These filters were used for samples taken over the periods 0–12h and 24–30h. A suitable volume of fresh medium was added to the culture and growth allowed to proceed for a further 12h, by which time the cell density had regained the original value of 2×10^6 – 3×10^6 myxamoebae/ml of medium.

Fig. 1. Specific activity of β -N-acetylglucosaminidase during differentiation of *D. discoideum* myxamoebae grown under different conditions

(a) ●, Myxamoebae grown on bacteria, plated out at 5×10^7 myxamoebae/Millipore filter; ■, myxamoebae grown on axenic medium and harvested when in the stationary phase of growth; ▲, myxamoebae grown on axenic medium+glucose (86mm) and harvested when in the stationary phase of growth. (b) ○, Myxamoebae grown on bacteria, plated out at 10^8 myxamoebae/Millipore filter; □, myxamoebae grown on axenic medium and harvested when in the exponential phase of growth; △, myxamoebae grown on axenic medium+glucose (86mm) and harvested when in the exponential phase of growth.

Millipore filters were then prepared as before at a cell density of 5×10^7 myxamoebae/filter and were used for samples taken over the period 12–24 h. This procedure ensured that, despite variations in enzyme amount during the growth phase (Ashworth & Quance, 1972), the two samples of myxamoebae had similar enzymic compositions. The complete cell differentiation process takes 24 h under these conditions (Sussman, 1966) and to obtain a complete set of samples two batches of myxamoebae were obtained as described above. Cells were harvested into 3 ml of water and the suspensions were stored frozen before being assayed for protein and for various enzyme activities. Duplicate samples of myxamoebae from the stationary phase of growth were obtained in the same fashion but harvested when at 6×10^6 – 8×10^6 myxamoebae/ml of medium for myxamoebae grown on axenic medium and at 2×10^7 – 3×10^7 myxamoebae/ml when the axenic medium also contained 86 mM-glucose. Millipore filter samples were prepared as described above from these myxamoebae and were used to provide samples over the time periods 0–12, 24–30 and 12–24 h.

Results

Myxamoebae of *D. discoideum* Ax-2 that have been grown in axenic medium and are then harvested in the exponential or stationary phases of growth form fruiting bodies (i.e. differentiate) in the same fashion and follow the same morphogenetic sequence as when they are grown on bacteria (Watts & Ashworth, 1970). The only visible differences are in the size and density of fruiting bodies formed by similar numbers of myxamoebae deposited at similar cell densities on Millipore filter supports. Myxamoebae grown on axenic medium lacking added carbohydrate form approximately three times as many fruiting bodies as those grown on medium containing carbohydrate. The fruiting bodies are, in consequence, smaller than those formed by myxamoebae grown on axenic medium containing 86 mM-glucose, and these are similar in size and density to those formed by myxamoebae grown on bacteria. This is true whether the axenically grown myxamoebae are harvested when in the exponential or stationary phases of growth and presumably reflects differences in the

Fig. 2. Specific activity of α -mannosidase during differentiation of *D. discoideum* myxamoebae

The conditions for (a) and (b) and the symbols are the same as in Fig. 1.

Fig. 3. Specific activity of β -glucosidase during differentiation of *D. discoideum* myxamoebae grown under different conditions

The conditions for (a) and (b) and the symbols are the same as in Fig. 1.

Fig. 4. Specific activity of acid phosphatase during differentiation of *D. discoideum* myxamoebae grown under different conditions

The conditions for (a) and (b) and the symbols are the same as in Fig. 1.

Fig. 5. Specific activity of alkaline phosphatase during differentiation of *D. discoideum* myxamoebae grown under different conditions

The conditions for (a) and (b) and the symbols are the same as in Fig. 1. Figs. 1–5 represent one experiment; in other experiments with alkaline phosphatase the deviations of the ● and ▲ points from the single line (Fig. 5a, 5b) were much less, or non-existent.

aggregation behaviour. However, as the morphogenetic sequence apparently remains unaffected by the size of the aggregate, their existence does not affect the argument presented in this paper.

In Figs. 1–5 the variation in specific activity of a number of enzymes is shown during the morphogenetic sequence characterizing cell differentiation in the cellular slime mould. Zero time (0h) is when the myxamoebae are deposited on the Millipore filter supports and the fruiting bodies are completely formed by 22–24h in all cases.

Myxamoebae grown axenically will only differentiate synchronously to this time-schedule if they are deposited on the Millipore filter supports at a cell density of $<5 \times 10^7$ myxamoebae/filter, whereas when grown on bacteria a cell density of 10^8 myxamoebae/filter is suitable. All previous studies on the enzymes described in Figs. 1–5 have been done with the NC-4 strain grown on bacteria and allowed to differentiate at a cell density of 10^8 myxamoebae/filter. Further,

when grown on bacteria, myxamoebae have a lower protein content and are smaller than when they are grown axenically, so it is not clear what experimental conditions are appropriate for bacterially grown cells if comparison is to be made with axenically grown cells. For this reason we present results in Figs. 1–5 for bacterially grown cells at densities of both 5×10^7 and 10^8 myxamoebae/filter. This has no qualitative effect on the pattern of enzyme synthesis but significant quantitative differences are seen with α -mannosidase and *N*-acetylglucosaminidase synthesis.

Discussion

An enzyme can be considered to be developmentally regulated, and thus its synthesis to be controlled by the 'developmental programme', when:

1. Its specific activity rises significantly (usually

taken to be more than threefold) during the cell differentiation process.

2. This increase in specific activity is aberrant in morphologically deranged mutants.

3. This increase in specific activity is affected by known inhibitors of protein synthesis (usually cycloheximide and actinomycin D).

4. This increase in specific activity is affected by mechanical perturbation of the normal morphogenetic sequence.

Of the enzymes shown in Figs. 1–5, α -mannosidase (Loomis, 1970), β -*N*-acetylglucosaminidase (Loomis, 1969*a*), alkaline phosphatase (Loomis, 1969*c*) and β -glucosidase (Coston & Loomis, 1969) satisfy criteria 1–4 when bacterially grown *D. discoideum* NC-4 are studied. Acid phosphatase (Fig. 4) has not been studied so extensively and is included here because it resembles α -mannosidase, β -*N*-acetylglucosaminidase and β -glucosidase in being located in the lysosomal fraction (Ashworth & Quance, 1972; Wiener & Ashworth, 1970). Of other developmentally regulated enzymes, UDP-glucose pyrophosphorylase (which satisfies criteria 1–3; Ashworth & Sussman, 1967) has been shown to be synthesized in a similar fashion in myxamoebae grown axenically in a glucose-containing medium or when grown on bacteria (Ashworth & Watts, 1970), despite the initially rather different concentrations of the enzyme that these myxamoebae contain (Edmundson & Ashworth, 1972). Also, UDP-galactose-polysaccharide transferase (which satisfies criteria 1–4; Sussman & Sussman, 1969) synthesis has been shown to be identical in differentiating myxamoebae whether these myxamoebae are grown on bacteria or in an axenic medium containing glucose (Schwalb & Roth, 1970).

The results reported here for the synthesis during differentiation of enzymes by bacterially grown *D. discoideum* Ax-2 myxamoebae agree qualitatively with results reported (Loomis, 1969*a,c*, 1970; Coston & Loomis, 1969) for bacterially grown myxamoebae of *D. discoideum* NC-4. Further, in all cases except alkaline phosphatase, there is also extremely good quantitative agreement between our results and those reported for strain NC-4. We find values for alkaline phosphatase specific activities of all stages that are approximately twice those reported by Loomis (1969*c*). This discrepancy is probably caused by the inclusion of Triton X-100 in all our assays and this detergent is known to activate one of the alkaline phosphatases present (Ashworth & Quance, 1972). It seems, therefore, that the 'developmental programme' for the two strains is identical when they are both grown on bacteria.

Consideration of the results presented in Figs. 1–5 and those reported by others above shows that it is possible to divide the 'developmentally regulated' enzymes into three classes. First, there are those

enzymes (alkaline phosphatase, UDP-glucose pyrophosphorylase and UDP-galactose-polysaccharide transferase) the synthesis of which is essentially unaffected by changes in the growth medium of the myxamoebae. Secondly, there are those enzymes (acid phosphatase and β -glucosidase) the synthesis pattern of which remains the same, but for which there are quantitative differences that depend on the nature of the growth medium of the myxamoebae. Finally, there are those enzymes (α -mannosidase and β -*N*-acetylglucosaminidase) that show both qualitative and quantitative changes in enzyme synthesis, depending on the growth medium used for the myxamoebae. A given morphogenetic stage can thus contain a variety of enzyme assemblies, depending on the origin of the myxamoebae from which it is derived. Similar conclusions have been reached by Newell & Sussman (1970) on the basis of rather different types of experiment.

It would seem reasonable, therefore, to conclude that the 'developmental programme' is concerned with regulating the flux of metabolites through the biochemical pathways of the differentiating cell so that the pattern of macromolecular biosyntheses is altered. An alteration in the concentration of a particular enzyme might be one mechanism or part of the mechanism that changes a particular flux but such alterations in enzyme concentration are clearly only the means of effecting the change, and are not the primary requirement for homeostasis in themselves. The view that cell differentiation is essentially a process of selective gene activation is thus a partial and incomplete definition of the problem and the 'developmental programme' must involve events other than the sequence in which genes are activated and the amount of product(s) that are produced from such genes. In order to determine what these other events are it is necessary to study a metabolic pathway in which it is possible to determine the flux through the pathway, as well as the intracellular concentration of the relevant enzymes and their substrates and effectors. We have shown that it is possible to alter the glycogen level of the myxamoebae during the growth phase (Ashworth & Watts, 1970) and it is known (White & Sussman, 1961) that during the differentiation of bacterially grown myxamoebae carbohydrates (particularly trehalose and cellulose) are newly synthesized whilst the glycogen content of the myxamoebae alters. The metabolic pathways leading to these carbohydrates are well known and have comparatively few unique steps so that this area of metabolism might be one in which it is possible to investigate the relative importance of the various factors that might regulate the changes in metabolic flux that occur during cell differentiation. In the next paper (Weeks & Ashworth, 1972) we describe the factors that regulate glycogen synthesis during growth of the myxamoebae.

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