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Excretion and Accumulation of Certain Developmentally Regulated Enzymes due to *p*-Fluorophenylalanine Incorporation in *Dictyostelium discoideum*

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Metabolic patterns in *Dictyostelium discoideum* are reflected in morphological differentiation. Treatment of myxamoebae with p-fluorophenylalanine (FPA), an analogue of phenylalanine, under buffer-shaken conditions, reduces the excretion of β-N-acetylglucosaminidase, α-mannosidase, α-glucosidase and β-glucosidase. Consequently, FPA-treated cells show higher activities of these enzymes as compared to the untreated cells. Inhibitory effects of FPA in *D. discoideum* appear to be due to its incorporation into most, if not all, of the proteins synthesised during the period of treatment. Thus, with FPA it is possible to affect selectively and more or less quantitatively the activity of a set of enzymes, synthesised during a particular period of growth or development.

Key Words : **Enzymes,** *Dictyostelium*, **Development, Slime mould, Differentiation**

Introduction

Structural analogues of essential metabolites have been frequently and successfully used as metabolic probes. p-Fluorophenylalanine (FPA), an analogue of phenylalanine, has been shown to retard growth and cause abnormal morphogenesis in the cellular slime mould, *Dictyostelium discoideum* (Sinha & Ashworth 1978). In this paper we describe the effects of FPA on the excretion into the medium and accumulation in the cell of certain developmentally regulated enzymes of this slime mould.

The life cycle of *D. discoideum* consists of two mutually exclusive phases—the first growth phase during which the individual myxamoebae multiply by binary fission, and the second, differentiation phase during which the myxamoebae aggregate and differentiate to form the fruiting bodies. Thus, it is possible to study the morphogenetic behaviour of two physiologically distinct but genetically identical cell populations (Garrod & Ashworth 1972). The effects of FPA on differentiation in *D. discoideum* are to a great extent irreversible (Sinha & Ashworth 1978). Patterns of biochemical events in this mould, particularly the appearance and disappearance of

various enzymes and their excretion and accumulation during different stages of growth and differentiation have been extensively studied by various workers (Sussman & Sussman 1969, Garrod & Ashworth 1972, Loomis 1975). But a detailed analysis of interactions between various stages in the developmental programme, as expressed by such changes in enzymic activities, has been hampered by the lack of a technique to inhibit the activity or synthesis of a particular enzyme or a group of enzymes. Addition of FPA provides some hope of achieving this, because if different enzymes are synthesised during different periods, FPA can be given for a short period, thus rendering ineffective a particular enzyme or a set of enzymes without affecting the preceding or succeeding ones.

Materials and Methods

Organism: D. discoideum strain Ax-2 (ATCC 24397) cells were grown at 22°C in liquid axenic cultures as described by Watts and Ashworth (1970).

Differentiation on Millipores: Cells were harvested, washed and resuspended in cold distilled water at a density of 5×10^7 cells/ml. 0.5 ml of this suspension was spread on Millipores or support pads, saturated with pad diluting fluid (PDF) (Sussman 1966). PDF contained p-fluorophenylalanine or phenylalanine, whenever necessary. Filters contained in petridishes were incubated at 22°C in a dark and humid chamber. Under normal conditions mature fruiting bodies are formed after 24 hr (Sussman 1966).

Enzyme assays: Methods of assaying β-N-acetylglucosaminidase, α and β-glucosidases and α-mannosidase have been described by Every and Ashworth (1973). One unit of enzyme activity is defined as the amount of enzyme required to hydrolyse 1n mole of the substrate per min at 35°C.

Electrophoresis: Glucose grown Ax-2 cells were harvested at a cell density of 1×10^6 cells/ml, washed in cold water and resuspended in 16.6 M phosphate buffer (*p*H—6.0) at 1.5×10^7 cells/ml. Exactly 0.8 ml of such a cell suspension was shaken for 1 hr at 22°C and then 0.1 ml of 50 mM FPA and 10μc of^{14}C-FPA were added. The mixture was shaken for another 5 hr, harvested, washed in cold water and the pellet was frozen in a dry ice - ethyl alcohol freezing mixture. The frozen pellet was heated to 100°C for 10 min after mixing with 0.15 ml water, 0.02 ml of 10% sodium dodecyl sulphate (SDS) and 0.0005 ml of 10% mercaptoethanol. Three duplicate 10% SDS / 7% acrylamide gels were run with loadings of 500 μg protein per gel. After separation, the gels were stained with Coomassie Blue, scanned at 550 nanometers with a Joyce-Loebel Transtab (type D8) chromoscan, frozen and cut into 1 mm slices. The radioactivity of each slice was determined according to the method of Basch (1968), using a Packard scintillation counter.

Chemicals: ^{14}C-p-Fluorophenylalanine (specific activity 5.9 mCi/mM) was obtained from New England Nuclear Corporation, Boston, USA. Other chemicals were obtained from BDH, Nottingham, England and were of analytical grade.

Results and Discussion

Effects of FPA on certain developmentally regulated enzymes: FPA affects the process of morphogenesis, and differentiation is the outcome and net result of enzymic functions and interactions. Quite a few enzymic activities are known to appear and disappear during development (Loomis 1975). The effect of FPA on the specific activities of four enzymes was investigated. Millipores incubated in the presence of FPA (40 mM)

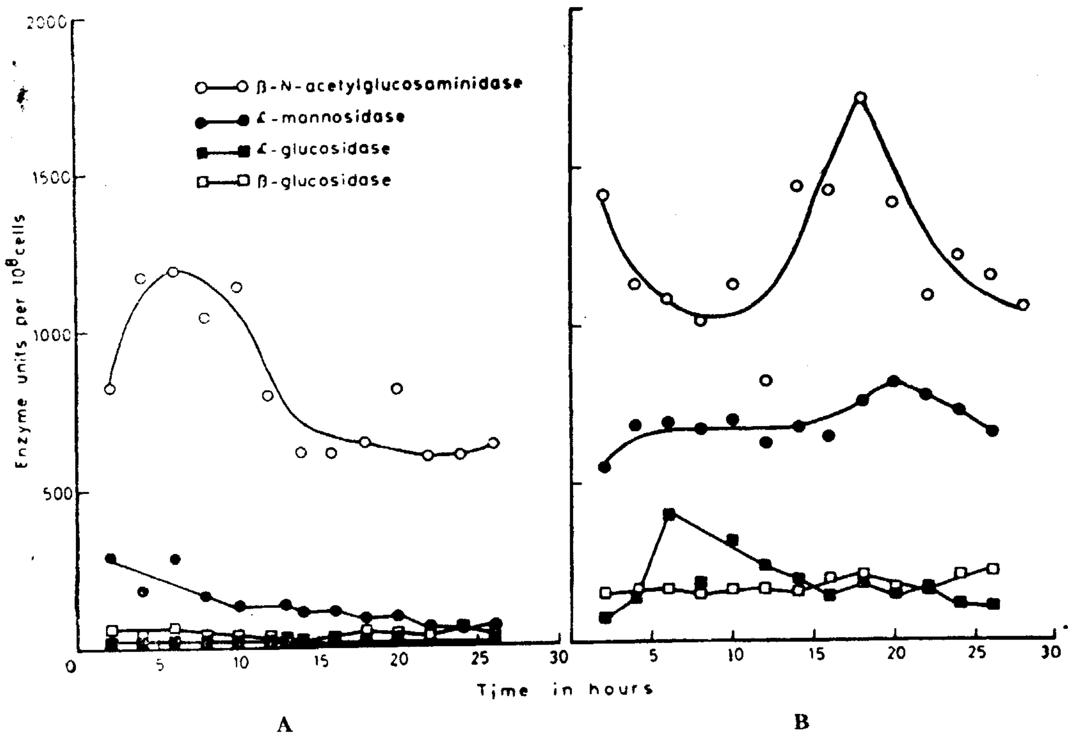

Figure 1 Changes in the cellular contents of four developmentally regulated enzymes during the development of *D. discoideum* cells on Millipore filters in the absence (A) and presence (B) of 0.04 M p-fluorophenylalanine. Each point represents a mean of three readings.

were harvested at different time intervals, and specific activities of various enzymes determined. Results summarised in figures 1A and 1B indicate considerable differences between treated and untreated cells. In untreated cells, there is maximum specific activity of α-mannosidase and β-N-acetylglucosaminidase during the first 4–10 hr of differentiation. But in treated cells, the peak specific activity appears after 18–20 hr. At the same time total specific activities of all the enzymes studied were found to be higher in the FPA treated cells than in the untreated ones. Phenylalanine treated cells showed enzymic activities like the untreated cells. As reported earlier (Sinha & Ashworth 1978), 40 mM of FPA slows down the process of differentiation on millipores, which is finally arrested at the Mexican hat and pseudoplasmodial stage. Rippling and aggregation start only after about 14 and 17 hr, respectively. Aggregates are formed, disrupted and reformed during 19 through 25 hr and by 27 hr early pseudoplasmodia are discernible. On the other hand, during normal differentiation in the controls, rippling starts after 5 hr of the deposition of myxamoebae on the millipores, aggregation occurs between 6 through 10 hr, pseudoplasmodia are formed by 15 hr, Mexican hats by 19 hr and mature fruiting bodies by 25 hr.

If cells are harvested from growth medium and then shaken in sodium phosphate buffer (16.6 mM and *p*H 6.0) for up to

6 hr and then dispensed on solid supports, the time taken to form fruiting bodies is shortened by any time equal to the time of incubation in buffer, suggesting that the metabolic changes that occur during the early stages of differentiation on a solid support, can also take place in buffered suspensions (Gerisch 1968, Lee 1972, Sinha 1974). The activities of the four enzymes, therefore, were measured in the presence and absence of FPA, under buffer-shaken conditions, in cells as well as growth media. The results of such experiments indicate that the rates of accumulation of most of the glycosidases remain constant for 6 hr in buffer, but considerable amounts of β-N-acetyglucosaminidase and α-glucosidase are excreted by the cells under normal conditions (Every & Ashworth 1975). This excretion is reduced to about one third of the normal in FPA-treated cells. Consequently, β-N-acetylglucosaminidase activities are higher in FPA-treated cells and show peaks at about 2 hr and 16 hr. These studies indicate that the whole process of morphogenesis is slowed down in the presence of this analogue. It appears that the enzymes studied during the present investigation are synthesised before aggregation starts but they become active only during differentiation. There is a need, therefore, to study the effects of FPA on an enzyme which is synthesised and expressed during aggregation or the subsequent stages of differentiation.

Incorporation of FPA into proteins: FPA is an analogue of phenylalanine and, therefore, in many organisms is incorporated into proteins in the place of phenylalanine residues. In order to find out whether this is also the case with *D. discoideum* and whether this incorporation is random, the myxamoebae were grown in the presence of ^{14}C-FPA, and the total cellular protein analysed by SDS/acrylamide gel electrophoresis. Figure 2 shows that FPA is incorporated into most, although not quite all the proteins. It could well be that those bands which do not show appreciable amounts of

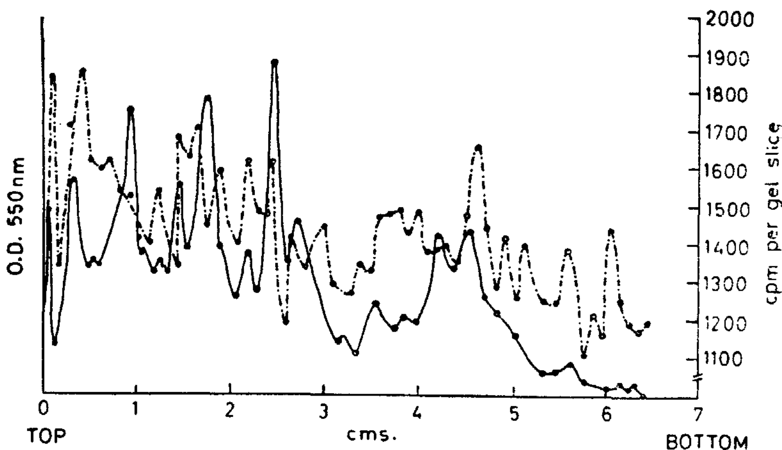

Figure 2 Incorporation of ^{14}C-p-fluorophenylalanine into the proteins of *D. discoideum* myxamoebae separated by gel electrophoresis. Continuous line represents the OD_{550} nm trace and the broken line represents the radioactivity in 1 mm gel slices.

radioactivity, contain proteins or polypeptides which were synthesised before the addition of FPA or which have fewer phenylalanine residues.

The phosphatases of *Aspergillus nidulans* have been shown to be inactivated due to FPA incorporation (Singh & Sinha 1979). In many instances FPA acts by repressing, derepressing or inhibiting certain enzymes (Previc & Binkley 1964, Knorre et al. 1971). But the present studies clearly indicate that ^{14}C-FPA is incorporated into most, although not all of the proteins. Radioactivity in the FPA molecule is in such a place that its degradation during this short exposure is very unlikely. Present studies do not indicate whether the activity of enzyme/enzymes is proportionately reduced due to increasing incorporation of FPA into the proteins of *D. discoideum*.

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