THE METABOLIC CONTROL OF DEVELOPMENT IN THE CELLULAR SLIME MOULD DICTYOSTELIUM DISCOIDEUM

by

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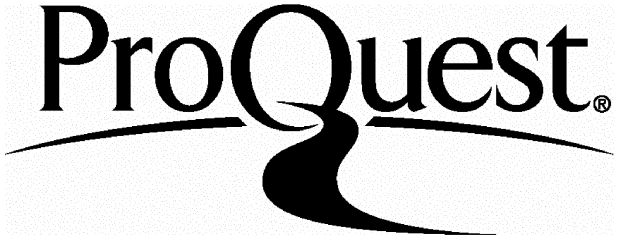

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DEFINITIONS

Axenic Medium

An axenic medium, as referred to in this thesis, is one which lacks the presence of any organism other than myxamoebae of *Dictyostelium discoideum* which were inoculated into it.

Normal Development

For the purposes of this thesis, the development of *Dictyostelium discoideum* strain NC-4, grown on a lawn of *Aerobacter aerogenes* or *Escherichia coli* as described by Sussman (1966), is taken to be representative of normal development in *Dictyostelium discoideum*.

End-product Saccharide

During the developmental phase of *Dictyostelium discoideum*, certain carbohydrates are synthesised which, once formed, are not degraded until spore germination, and hence these have been termed end-product saccharides.

Cell Wall Polysaccharide

Spores and stalk cells of mature fruiting bodies (sorocarps) of *Dictyostelium discoideum* are encased in a material comprising equal parts by weight of cellulose-like and glycogen-like polymers (Ward & Wright, 1965), the latter being clearly distinguishable from cellular cytoplasmic (alkali soluble) glycogen by being insoluble in alkali (White & Sussman, 1961). The cellulose-glycogen complex is referred to in this thesis as cell wall polysaccharide.

ABBREVIATIONS

G6P	=	glucose 6-phosphate
G1P	=	glucose 1-phosphate
T6P	=	trehalose 6-phosphate
ADPG	=	adenine diphosphoglucose
CDPG	=	cytidine diphosphoglucose
GDPG	=	guanosine diphosphoglucose
UDPG	=	uridine diphosphoglucose
FDPase	=	fructose 1,6-diphosphatase
T6P synthase	=	trehalose 6-phosphate synthase
CO_2	=	carbon dioxide
TCA	=	trichloroacetic acid
PDF	=	pad diluting fluid
BSA	=	bovine serum albumin
KOH	=	potassium hydroxide
NaOH	=	sodium hydroxide
H_2SO_4	=	sulphuric acid
NaCl	=	sodium chloride
KCl	=	potassium chloride
$MgCl_2$	=	magnesium chloride

All other abbreviations used in this thesis are as recommended by The Biochemical Journal (1972) 126, 1.

GENERAL INTRODUCTION

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One of the greatest enigmas of modern biology concerns the mechanisms whereby a fertilised unicellular ovum transforms itself into a complex, organised, multicellular individual, the component cells of which differ both structurally and functionally. This paradox of cellular heterogeneity in the presence of identical genetic information has prompted investigations into all aspects of gene expression. Whilst such studies have not been without success, it is true that our knowledge of the mechanisms which operate to modify the cellular phenotype is still elementary.

Attempts to understand the means whereby such cellular phenotypic variation is achieved have relied on simplification of the attendant problems. Thus, Huxley (1924) realised that embryonic development could be considered as consisting of two separate but related phases; in the primary phase, the presumptive fate of the cell is determined although there are no detectable morphological changes, and, in the secondary phase, the cell undergoes morphological changes that result in an altered phenotype.*

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* This distinction between a phase of determination followed by a phase of differentiation will be recognised throughout this thesis; the word development will be used "to include all the morphological and biochemical events that are involved in the construction of the (slime mould) fruiting body, the word differentiation will be reserved for that part of development concerned with the elaboration of specialised cell types such as the stalk cells and spores from amoebae" (Newell, 1972).

Experimentally, simplification has been achieved by the use of primitive eukaryotic cell systems which, whilst exhibiting developmental changes, lack many of the experimental difficulties associated with higher eukaryotic cell systems. In recent years, studies of this kind using a group of organisms called the Acrasiales [or "cellular slime moulds" (Shaffer, 1953)] have proved extremely rewarding.

Originally discovered by Brefeld in 1869, the cellular slime moulds are eukaryotic soil protists. Dictyostelium discoideum, discovered by K.B. Raper in 1935, is the species most commonly used in developmental studies and offers many advantages to the experimentalist. The cells can be easily grown either in association with bacteria (Raper, 1940; Sussman, 1966) or in axenic culture (Sussman & Sussman, 1967; Schwalb & Roth, 1970; Watts & Ashworth, 1970) and, upon the exhaustion of external nutrients and termination of the growth phase, the vegetative cells embark on a phase of development which results in the formation of characteristic fruiting bodies (sorocarps), each consisting of a cellulose stalk supporting a mass of spores (Raper, 1935; Bonner, 1967) (Plate 1). Under rigorously defined experimental conditions, the developmental phase will occur in a highly synchronous manner within 24 hours. This, and the fact that only two major cell types are differentiated (spores and stalks) in the complete absence of the complications of cellular growth and division (Bonner & Frascella, 1952; Sussman & Sussman, 1960), make D. discoideum an extremely suitable system for the study of development. An additional advantage is that the vegetative cells are capable of genetic recombination (Sussman & Sussman, 1963)

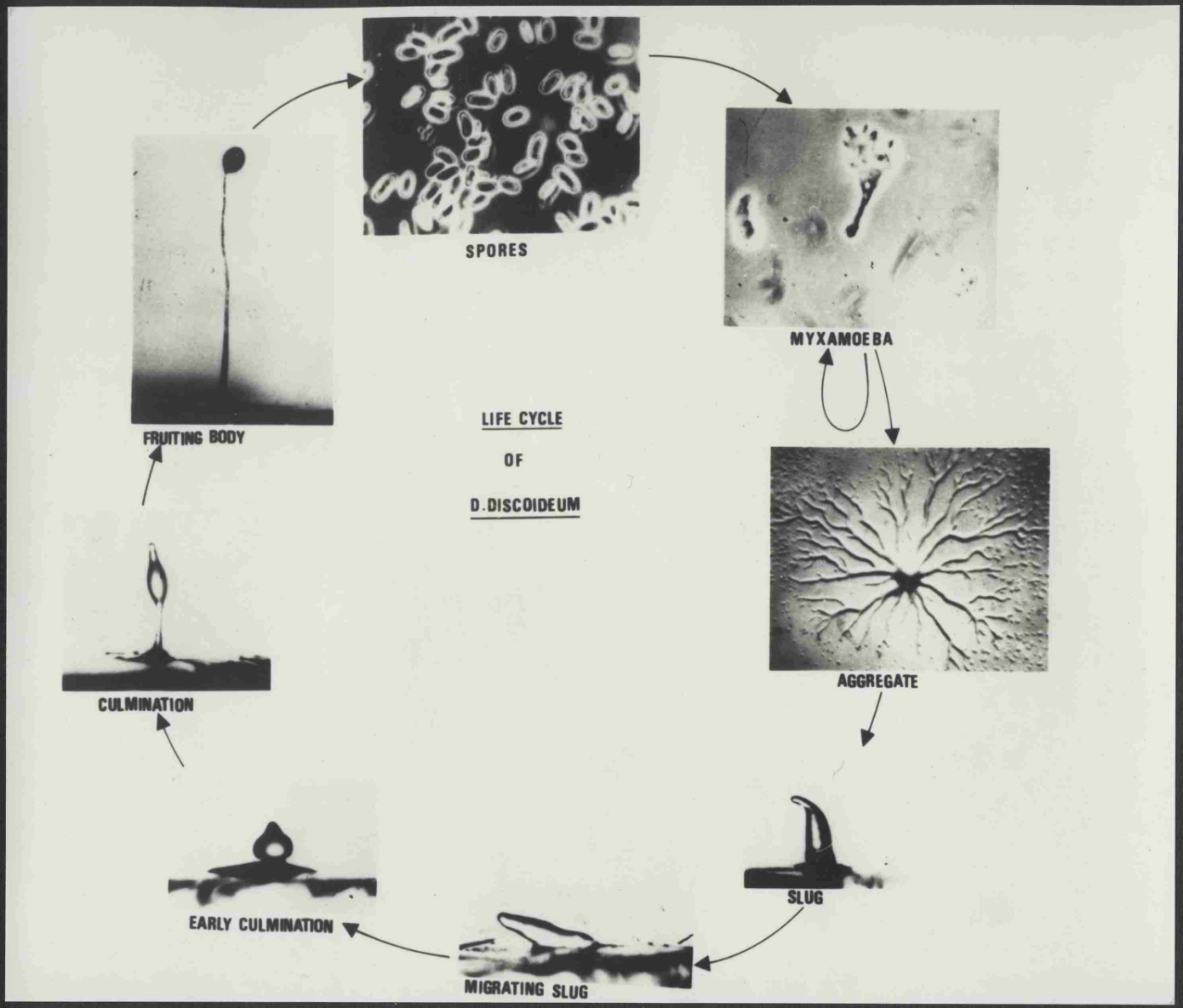

PLATE 1: The life-cycle of Dictyostelium discoideum.

by a parasexual process (Sinha & Ashworth, 1968) thus providing the potential for genetic definition of the developmental programme.

The remarkable transformation from solitary myxamoebae to multicellular fruiting body requires the formation of several different carbohydrates (Sussman & Sussman, 1969); indeed, carbohydrate metabolism is one of the major activities of the developing cells. This thesis investigates the biochemical controls which operate on carbohydrate metabolism during development and probes the relationship between carbohydrate, protein, and RNA metabolism in this organism.

CHAPTER I

THE AXENIC GROWTH AND DEVELOPMENT OF MYXAMOEBAE CONTAINING VARIOUS CELLULAR GLYCOGEN CONTENTS

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INTRODUCTION

Until 1967, developmental studies using the cellular slime mould, *Dictyostelium discoideum*, were carried out with myxamoebae grown on various bacterial species as food supply, usually *Aerobacter aerogenes* or *Escherichia coli*. These myxamoebae were found to undergo major biochemical changes during the developmental phase and these changes always occurred at the same times and to the same extents in the developmental phase. Such data, mainly derived from studies on the control of carbohydrate metabolism during development, led to the concept of a "developmental programme", that is, a genetically programmed series of biochemical events occurring during the developmental phase and responsible for successful development. Within this concept, biochemical changes which always occurred at the same times and to the same extents in the developmental phase, were referred to as being "developmentally regulated".

However, given that the chemical composition of bacterially-grown vegetative myxamoebae is largely invariant, it is not too surprising that the biochemical changes occurring during development are also invariant. Using such a system, it is not possible to distinguish essential developmental changes from those which are incidental and a function of the nature of the vegetative phase of the organism. One approach to this problem is to alter the growth conditions of the myxamoebae, and to examine the effect of such

alterations on the events which occur during development. Not only will studies of this kind help to identify genuine developmental changes but also, by providing a perturbation of the developing system, may give a better understanding of how essential developmental changes maintain their invariance under differing conditions.

Experimental modification of the growth conditions of vegetative D. discoideum myxamoebae was made possible by the acquisition of strains of the organism which could be grown in the complete absence of bacteria; that is, in complex, undefined, but axenic (p. iv)media (Sussman & Sussman, 1967; Watts & Ashworth, 1970; Schwalb & Roth, 1970; Cocucci & Sussman, 1970). The axenic strain of Watts & Ashworth (Ax-2) was especially promising since the myxamoebae could be grown in the absence or presence of glucose and, under such conditions, accumulated various cellular levels of glycogen, a key polysaccharide in the carbohydrate metabolism of developing D. discoideum myxamoebae (Wright et al., 1968b).

The first chapter of this thesis describes the development of reliable methods for obtaining vegetative myxamoebae containing various levels of glycogen and the effects of altered myxamoebal glycogen levels on the morphology and chronology of development.

MATERIALS

Bacteriological peptone and yeast extract were purchased from Oxoid Ltd., London; glucose, sucrose, ATP, $NADP^+$, hexokinase, glucose oxidase, and anthrone from Sigma (London) Ltd., London, U.K.; and glucose 6-phosphate dehydrogenase from Boehringer Corp. (London) Ltd., London W.5, U.K. All other chemicals were of the highest purity commercially available and were the products of BDH Chemicals Ltd., Poole, Dorset, U.K. or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

METHODS

Growth of Myxamoebae Myxamoebae of strain Ax-2 were grown axenically as described by Watts & Ashworth (1970). The basic axenic medium contained in 1 litre:

Oxoid bacteriological peptone (14.3g)
Oxoid yeast extract (7.15g)
$Na_2HPO_4 . 12\ H_2O$ (1.28g)
KH_2PO_4 (0.486g)

at pH 6.7

Glucose may be added to this basic medium as described under RESULTS

The culture conditions were either 60-90 ml of medium in a 250 ml Erlenmeyer flask or 700 ml of medium in a 2 litre Erlenmeyer flask, shaken on a rotary shaker with an eccentric rotation of 3 cm at 150-170 rev/min at 22-23°C air temperature. Stocks were maintained as unshaken 60 ml cultures.

Development of Myxamoebae Myxamoebae of strain Ax-2 were routinely harvested from the growth medium by centrifugation at 1000 g for 15 min at 0°C, and then washed by resuspension in an equal volume of ice cold 0.9% NaCl solution followed by centrifugation as above. The cell pellet was resuspended in ice cold water at a density of approximately 5-6 x 10^7 cells per ml and 0.5 ml samples placed on Millipore filter supports consisting of Millipore filters (cat. no. AABP 04700) resting on Millipore absorbant pads (cat. no. AP 100 4700) saturated with 1.6 ml of pad diluting fluid (PDF) (Sussman, 1966). The PDF used throughout these studies comprised 1.5g KCl, 0.5g $MgCl_2 \cdot 6 H_2O$, and 0.5g streptomycin sulphate per litre of 50 mM phosphate buffer pH 6.5. Development was then allowed to occur by incubation of the myxamoebae at 22-23°C in a humid environment in the dark.

Determination of Cellular Glycogen content Myxamoebae were harvested from growth media as described above and resuspended in ice cold water at a density of approximately 5 x 10^7 cells/ml. 2 ml of this suspension was used to isolate cellular glycogen, by the method of Cooper & Kornberg (1967), which was dissolved in 4 ml water and estimated by the anthrone assay of Hassid & Abraham (1957).

Determination of Total Cellular Hexose content Myxamoebae were harvested as described above, sonicated for 3 x 15 sec periods with continuous cooling in an ice-salt bath to prevent excessive heating, and samples assayed for hexose by the anthrone method of Hassid & Abraham (1957).

Electron Microscopy of Myxamoebae Myxamoebae, harvested and washed and resuspended in water as described above, were centrifuged at 2000 g for 10 minutes at 0°C and the cellular pellet resuspended in a solution of 2% glutaraldehyde in 0.2 M sucrose, 10 mM Tris-HCl buffer pH 7.6 for 2 h at 0°C. The fixed material was collected by centrifugation, post-fixed in buffered 2% osmic acid (30 min), dehydrated through an ethanol series and infiltrated with propylene oxide and Araldite, which was polymerised for 2 days at 60°C. Silver and grey sections were cut on a L. K. B. Ultratone III microtome and were stained with uranyl acetate.

The sections were examined in a Siemens Elmiskop IA electron microscope at an accelerating voltage of 80 kV with a 200 µm condenser aperture.

Photography of Sorocarps and Spores Sorocarps were photographed after 30 hours of development using a Leica MDA camera. Spores were removed from the tips of the sorocarps using a sterile loop, dispersed in 0.9% NaCl solution, and photographed within 15 min using a Zeiss Photomicroscope III camera.

RESULTS AND DISCUSSION

1. The Growth of Myxamoebae containing various glycogen levels

(a) Exponentially growing cultures

D. discoideum myxamoebae, strain Ax-2, grow in the basic axenic medium with a mean generation time of approximately 9 h, but this can be decreased to 8 h by the addition of glucose to the medium to 0.086 M final concentration (Fig. 1, from Watts & Ashworth, 1970). If the glucose concentration is increased beyond 0.1 M, decreased growth rates and cell yields result (Table 1).

Myxamoebae grown in the absence of added glucose contain low but variable levels of glycogen; over a three year period, values in the range of 0.046 - 0.310 mg glycogen per 10^8 myxamoebae have been obtained. The source of this variation is the Oxoid yeast extract used in the medium. Addition of glucose to the axenic medium increases the myxamoebal glycogen content in a linear manner over the range 0-0.3 M final medium glucose concentration (Fig. 2, Table 1), but further addition of glucose fails to increase the myxamoebal glycogen content beyond approximately 5.5 mg glycogen per 10^8 cells which appears to be the maximum myxamoebal glycogen content obtainable by this method.

Ashworth & Watts (1970) found that glycogen accounted for virtually all the carbohydrate material within myxamoebae grown in medium containing 0.086 M glucose, and concluded that the rate limiting factor in glucose utilisation under these conditions is the rate of transport of glucose across the plasma membranes of the myxamoebae.

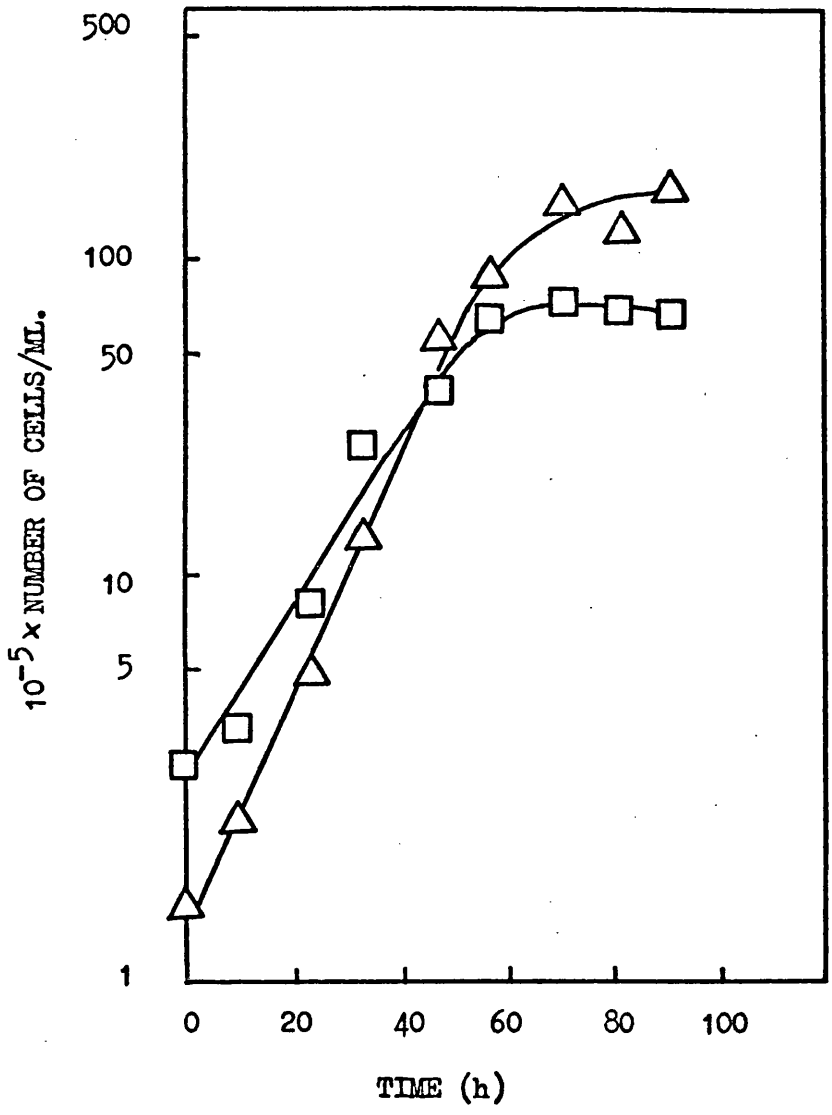

FIG.1 Growth kinetics of Dictyostelium discoideum Ax-2 myxamoebae.

□ ; growth of D.discoideum Ax-2 myxamoebae in axenic medium lacking added carbohydrate

△ ; growth of D.discoideum Ax-2 myxamoebae in axenic medium containing 86mM glucose

Each culture consisted of 700ml. medium in a 2l. Erlenmeyer flask shaken at 22°C on a rotary shaker and the increases in cell number were determined by haemocytometer counts.

(Figure taken from Watts @ Ashworth 1970)

TABLE 1 The Growth of Dictyostelium discoideum in Axenic Medium containing various concentrations of Glucose

Glucose Concentration in Medium (M)	Cell Doubling Time (h)	Cell Yield ($\times 10^6$ cells/ml)	Cellular Glycogen content (mg/10^8 cells)	Cellular Carbohydrate content (mg glucose equivs./10^8 cells)
0.000	10.5*	8.30 ± 0.74 (5)	0.11 ± 0.02 (12)	0.27 ± 0.03 (6)
0.086	8.0*	15.30 ± 0.55 (9)	1.69 ± 0.18 (8)	1.68 ± 0.10 (5)
0.100	9.83 ± 0.33 (3)	20.00 ± 0.00 (2)	1.70 ± 0.20 (2)	1.86 ± 0.026 (5)
0.200	13.67 ± 0.55 (9)	9.96 ± 2.63 (5)	2.78 ± 0.07 (3)	2.98 ± 0.07 (3)
0.300	19.67 ± 0.33 (3)	5.25 ± 0.75 (2)	4.77 ± 0.49 (4)	5.29 ± 0.33 (4)
0.400	29.50 ± 1.99 (5)	1.18 ± 0.35 (4)	4.25 ± 0.25 (2)	4.58 ± 0.08 (2)

Results are given as means ± standard error of the mean (S.E.M.)

Figures in brackets refer to the number of experiments

* Data taken from Ashworth & Watts (1970)

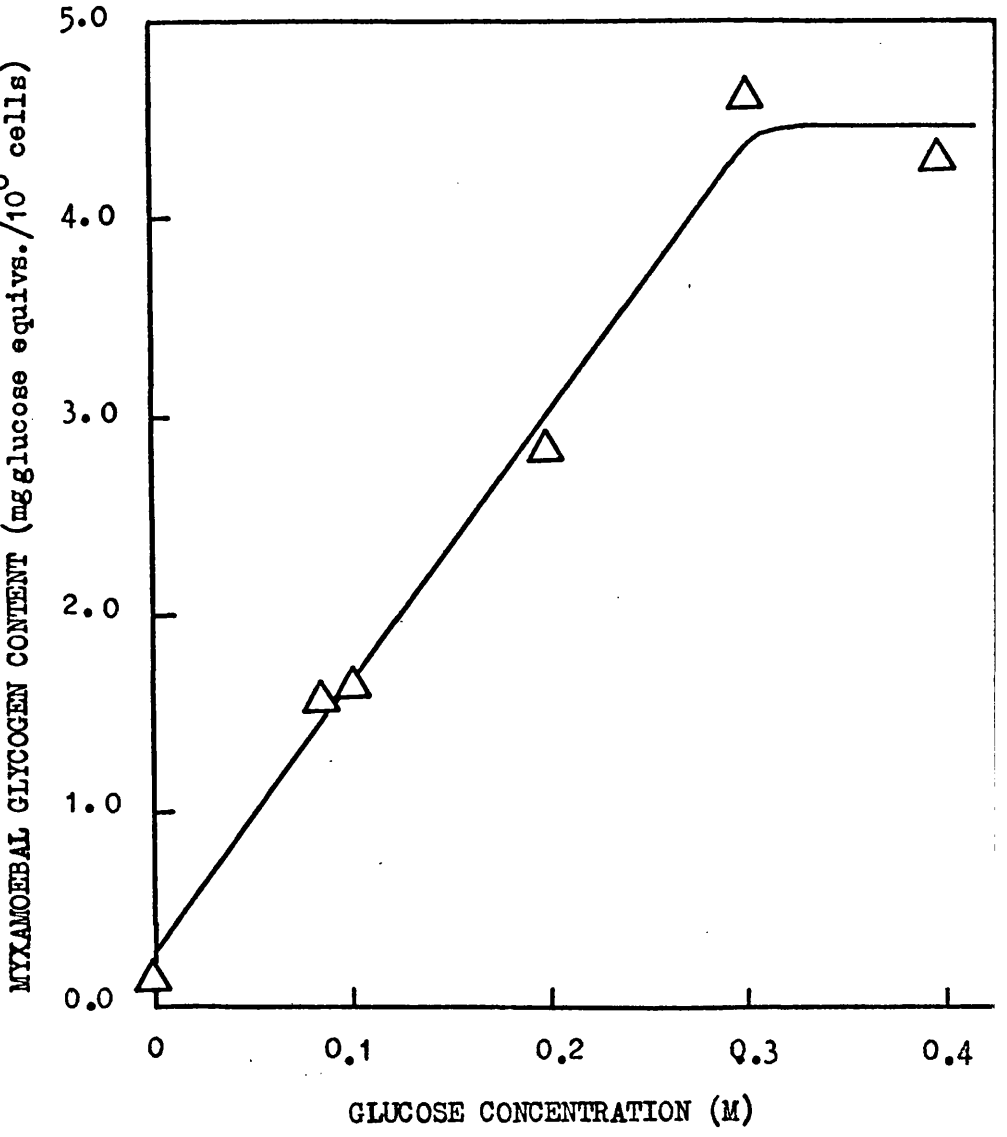

FIG.2 The Relationship between Myxamoebal Glycogen Content and The Concentration of Glucose in The Axenic Medium.

The data in Table 1 indicate that myxamoebae grown in any glucose concentration up to 0.4 M have greater than 90% of their carbohydrate as glycogen. Thus, presumably in these cells also, glucose transport is the rate limiting step in glucose utilisation.

However, Weeks & Ashworth (1972) showed that myxamoebae grown in axenic medium containing 0.086 M glucose, possess increased intracellular levels of UDP-glucose, the substrate of glycogen synthetase, and of G6P, an effector of glycogen synthetase that relieves ATP inhibition of the enzyme. No difference in total cellular glycogen synthetase activity was observed between myxamoebae grown in the presence or absence of added glucose and the difference in glycogen degradative rates between the two sets of cells was insignificant. Therefore, the increased glycogen contents of myxamoebae grown in the presence of added glucose are probably caused by increased intracellular levels of metabolites, suggesting that, although myxamoebae are not readily permeable to extracellular glucose, the rate limiting step in glycogen accumulation is glycogen synthetase itself.

(b) Near-Stationary Phase Cultures

Weeks & Ashworth (1972) showed that during exponential growth, the cellular glycogen content remains constant whether cells are grown in the presence or absence of added glucose. However, in medium containing 0.086 M glucose, there is an increase in the cellular glycogen content when the growth rate begins to decrease at the end of exponential growth (Fig. 3). The glycogen content reaches a maximum in early stationary phase and later decreases. No corresponding changes occur in the glycogen content of cells in medium that does not

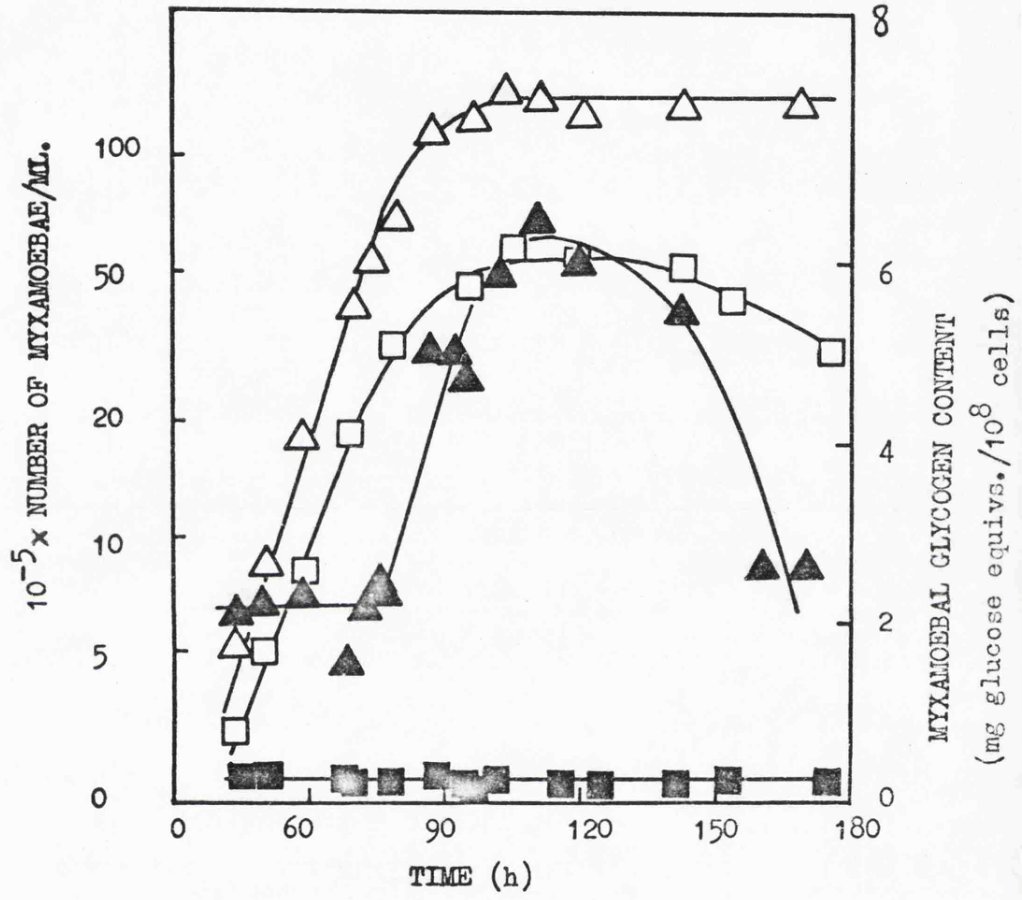

FIG.3 Myxamoebal Glycogen Content at Various Times during Growth.
Myxamoebae of D.discoideum strain Ax-2 were grown axenically and samples removed at the indicated times.Cell density was determined by counting cell number with a haemocytometer.

□ ; myxamoebae grown in axenic medium in the absence of glucose.

△ ; myxamoebae grown in axenic medium in the presence of 86mM glucose.

■ ; glycogen content of myxamoebae grown in the absence of glucose.

▲ ; glycogen content of myxamoebae grown in the presence of 86mM glucose.

(Figure taken from Weeks @ Ashworth 1972)

contain added glucose. Furthermore, Weeks & Ashworth (1972) showed that the increase in glycogen content of cells grown in 0.086 M glucose-containing medium and approaching stationary phase is not caused by an increase in the glycogen synthetic capacity of the cell nor by a decrease in the glycogen degradation rate. Thus the increase in glycogen content is presumably caused by variations in intracellular metabolite concentrations as described above for exponential phase myxamoebae.

The studies described in this thesis have used myxamoebae with high glycogen contents derived by either of the above methods. Whilst no differences have been observed that are attributable to the source of these cells, the second method described is preferred since greater cells yields are possible within much shorter time periods.

Location of Myxamoebal Glycogen

Electron microscopy of myxamoebae containing very low and very high glycogen contents (0.046 and 4.46 mg glycogen/10^8 cells respectively) reveals that the glycogen is located in the cell cytoplasm in granular form unbounded by membranes (Plate 2). Similar glycogen particles are found in other organisms (e.g. Vye & Fischer,1971).

2. The Development of Myxamoebae Containing Different Glycogen Levels

Bacterially-grown myxamoebae, when harvested and washed free of bacteria and plated on Millipore filter supports at a density of 10^8 myxamoebae per filter (Sussman, 1966) complete development within approximately 24 h in a synchronous fashion. However, axenically-grown myxamoebae, plated at this cell density, develop asynchronously

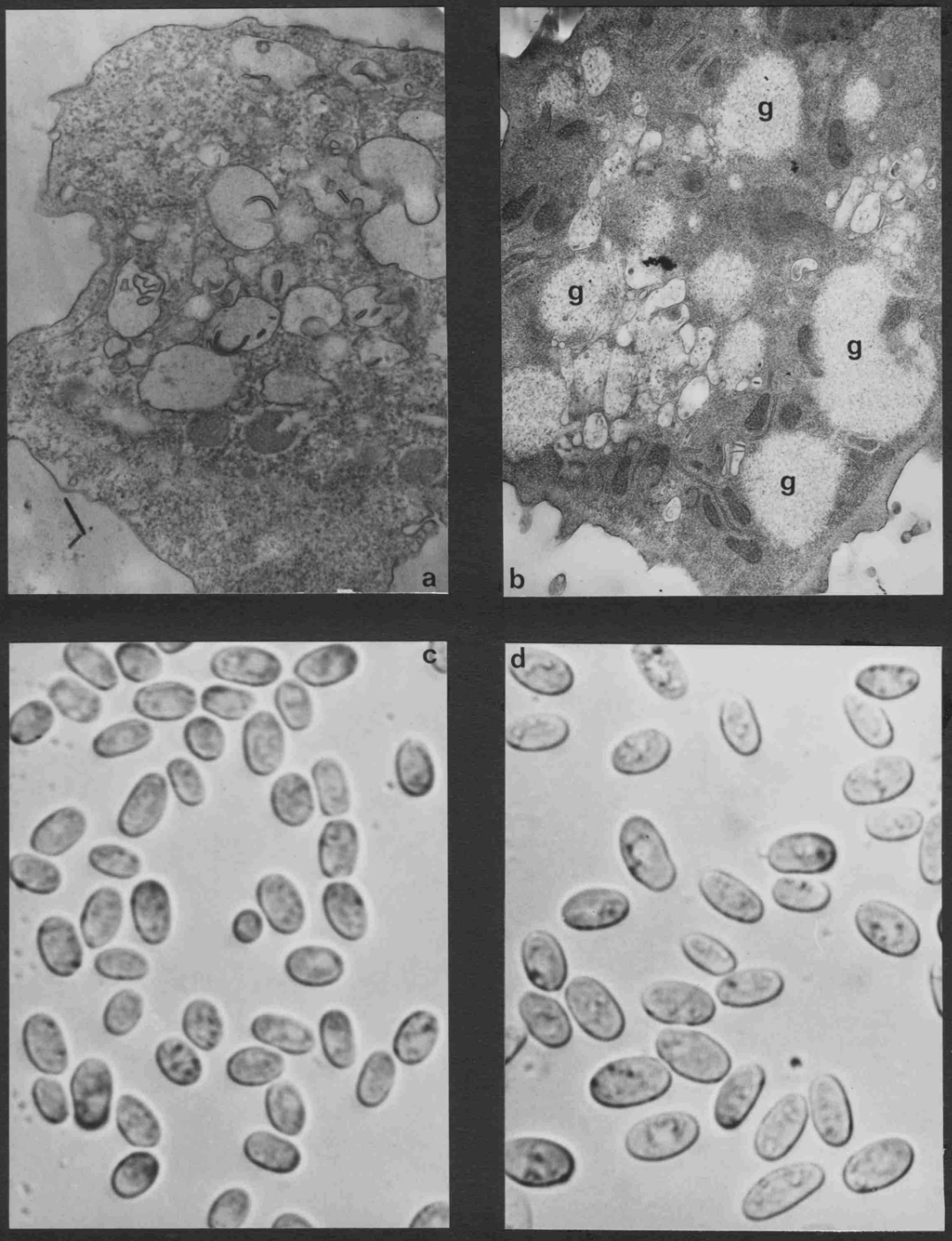

PLATE 2: The structure of D.discoideum Ax-2 myxamoebae grown in axenic media and the morphology of spores derived from the development of these cells.

a; electron micrograph (magnification ×12,600) of D.discoideum Ax-2 myxamoeba containing 0.046×10^{-8} mg glycogen.

b; electron micrograph (magnification ×12,600) of D.discoideum Ax-2 myxamoeba containing 4.640×10^{-8} mg glycogen.

c; photomicrograph (magnification ×1000) of spores derived from the development of myxamoebae initially containing o.13mg glycogen/10^8 cells.

d; photomicrograph (magnification ×1000) of spores derived from the development of myxamoebae initially containing 5.81mg glycogen/10^8 cells.

g; presumed location of myxamoebal glycogen.

and the resulting fruiting bodies are abnormal in appearance or not produced at all (Watts & Ashworth, 1970). Good synchrony of development and normal fruiting body morphology is achieved with axenically-grown cells only by lowering the cell density to 2.5×10^7 cells per Millipore support (Watts & Ashworth, 1970).

(a) Chronology of Development of Axenically-grown Myxamoebae

The time course for the appearance of the various stages involved in the formation of fruiting bodies from axenically-grown myxamoebae is to some extent dependent on the conditions of myxamoebal vegetative growth. Thus amoebae containing high levels of glycogen aggregate significantly earlier than those containing low levels of this carbohydrate, but the aggregate stage is more prolonged such that at 24 ± 1 h, fruiting bodies are formed by either set of cells (Fig. 4). This apparent dependence of chronology of development on myxamoebal glycogen content will be discussed in more detail in later chapters.

A few words of caution must be given here. If myxamoebae are allowed to remain in stationary phase for too long, instead of being harvested as they enter stationary phase, although they still develop when deprived of nutrients, the process appears to take longer to occur; aggregates may persist until 17-18 h developmental time and fruiting bodies may not be constructed until 27-28 h.

(b) Morphology of Development of Axenically-grown Myxamoebae

During the developmental phase of their life cycle, axenically-grown myxamoebae, irrespective of their glycogen content, exhibit morphological stages that are completely analogous to those

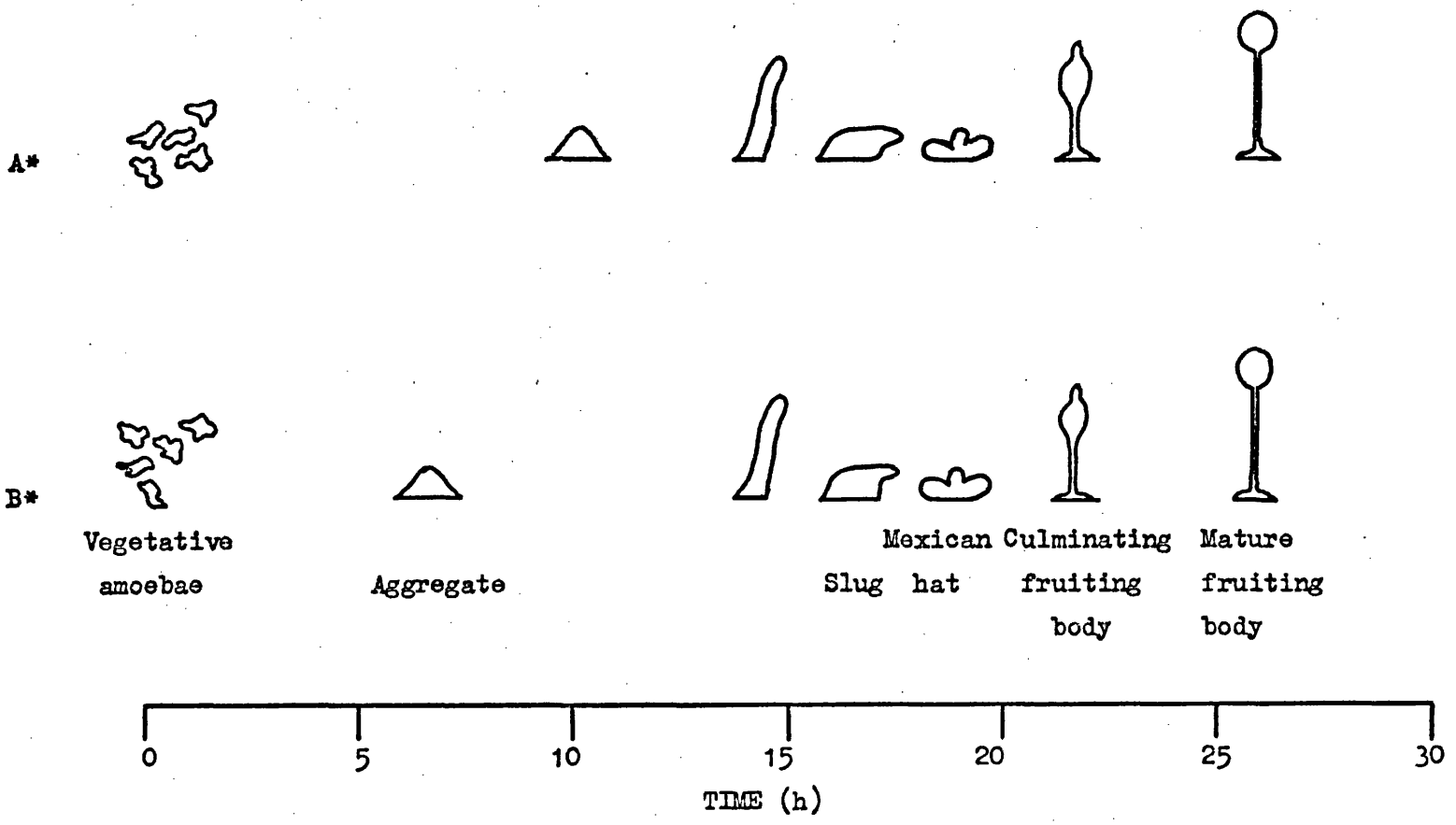

FIG.4 The Chronology of Development of Axenically-grown Myxamoebae of D.discoideum strain Ax-2.

A* ; axenically-grown myxamoebae initially containing less than approximately 3mg glycogen/10^8 cells.

B* ; axenically-grown myxamoebae initially containing more than approximately 3mg glycogen/10^8 cells.

observed during the development of bacterially-grown myxamoebae. Thus qualitatively, no differences are observed in the morphology of development which can be attributed to conditions of growth. However, there are striking quantitative differences in this respect.

Plate 3 compares the morphology of mature sorocarps produced from vegetative myxamoebae which differed only in their conditions of growth. Firstly, it is clear that myxamoebae grown in axenic medium produce smaller sorocarps than those grown on bacteria. But as the glycogen content of the axenically-grown myxamoebae is increased, by growth of the cells in axenic medium containing added glucose, so the fruiting body size increases, being compensated for by a decrease in the number of sorocarps produced. However, the effect of high cellular glycogen levels is not merely to increase sorocarp size; Garrod & Ashworth (1972) have shown that sorocarps produced from myxamoebae with a high glycogen content contain proportionally more spores than those produced from myxamoebae with low glycogen contents. Experiments on the sorting-out behaviour of axenically-grown myxamoebae also suggest that myxamoebae grown in conditions which induce glycogen accumulation have an increased tendency to form spores rather than stalk cells (D.R. Garrod, personal communication) and that the spores produced are approximately 40% larger in volume than those produced from myxamoebae containing low glycogen levels (Plate 2; Leach & Ashworth, 1972) whereas the myxamoebae giving rise to these spores differ by no more than 25% in volume (C.K. Leach, personal communication).

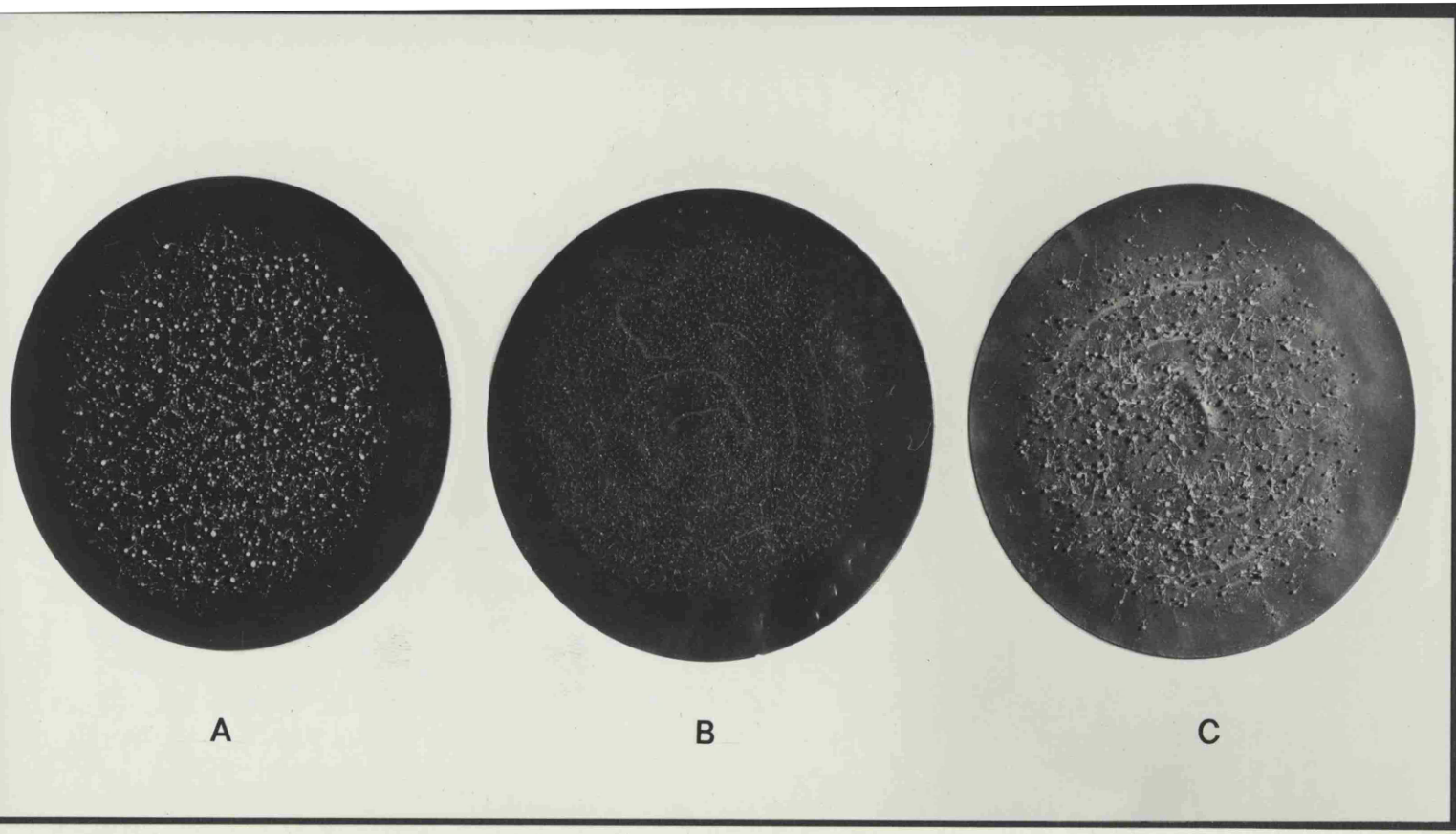

PLATE 3: The morphology of sorocarps derived from the development of myxamoebae grown under various culture conditions.

Myxamoebae were grown under the conditions specified below, harvested and allowed to develop on Millipore filters at a density of 5×10^7 cells/filter, and photographed as described in METHODS.

A; sorocarps derived from the development of D.discoideum Ax-2 myxamoebae grown on live Escherichia coli in liquid culture and harvested at a density of 3.2×10^6 cells/ml.

B; sorocarps derived from the development of D.discoideum Ax-2 myxamoebae grown in axenic medium lacking added glucose, harvested at a density of 2.5×10^6 cells/ml., and containing 0.31mg glycogen/10^8 cells.

C; sorocarps derived from the development of D.discoideum Ax-2 myxamoebae grown in axenic medium containing 86mM glucose, harvested at a density of 1.3×10^7 cells/ml., and containing 7.5mg glycogen/10^8 cells.

Summary

1. The glycogen content of the myxamoebae of the cellular slime mould D. discoideum is a function of both the composition of the growth medium and the phase of the growth cycle; the cellular glycogen content increases with increasing glucose concentration in the medium and with the entrance of cells into the stationary phase of the growth cycle.

2. Axenically-grown myxamoebae develop normally (p. iv) and synchronously within 24 $\pm$ 1 h irrespective of cellular glycogen content although aggregation occurs earlier and the aggregation stage persists longer in myxamoebae with a high glycogen content.

3. The size and cell composition of the mature sorocarp vary according to the myxamoebal glycogen content; high myxamoebal glycogen contents favour large sorocarps and spore rather than stalk cell formation.

CHAPTER II

THE METABOLISM OF GLYCOGEN DURING DEVELOPMENT

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INTRODUCTION

Research into the biochemistry of development of bacterially-grown myxamoebae has suggested that, during this process, the cells conserve (Wright & Dahlberg, 1967) or synthesise (White & Sussman, 1961; Sussman & Sussman, 1969) cellular glycogen which is then used during fruiting body construction, to synthesise at least three "end product" saccharides [trehalose, cell wall polysaccharide and an acid mucopolysaccharide, (p. iv)]. Indeed, based on *in vivo* measurements of metabolite pool sizes and *in vitro* measurements of enzyme kinetic parameters, Wright *et al.* (1968b) have formulated a computer model for the control of carbohydrate metabolism during this period, in which the synthesis and degradation of glycogen are key control events.

The glycogen content of axenically-grown myxamoebae of *D. discoideum* Ax-2 is a function of both the composition of the growth medium used (Ashworth & Watts, 1970; Chapter I) and the phase of the growth cycle (Weeks & Ashworth, 1972). In the preceding chapter, it has been described how, despite large variations in cellular glycogen content, the myxamoebae develop normally (p. iv) within the normal developmental time span to produce similar fruiting bodies and therefore in this chapter we investigate the role of glycogen during the development of axenically-grown myxamoebae.

MATERIALS

Trehalose and deoxyribose were obtained from Sigma (London) Ltd., London W.5, U.K.; phosphoglucomutase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase from Boehringer Corp. (London) Ltd., London W.5, U.K.; N-N-dioctylmethylamine from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; [U-^{14}C]glucose and [U-^{14}C]aspartate from The Radiochemical Centre, Amersham, Bucks., U.K.; and "Amberlite" Monobed MB3 resin from BDH Chemicals Ltd., Poole, Dorset, U.K. Dowex 1 (x8; A.G.; Cl^- form) and Dowex 50 (x8; A.G.; H^+ form) were obtained from Bio-Rad Laboratories, St. Albans, Herts., U.K. Dowex 1 (formate form) was prepared from Dowex 1 (Cl^- form).

All other chemicals were of the highest purity commercially available and purchased either from the sources described in Chapter I or from BDH Chemicals Ltd., Poole, Dorset, U.K.

METHODS

Growth and Development of Myxamoebae

Cells were grown, harvested and allowed to develop on Millipore filter supports as described in the preceding chapter.

Determination of DNA

At various times during the differentiation sequence, cells were harvested into ice cold water. Usually, sufficient cells were harvested to give a final suspension of 4×10^8 cells in 8 ml of water and these cell suspensions were then stored at -35°C. After being thawed, the cell suspensions were made up to 10 ml with water and 5 ml portions were sonicated with a 150W M.S.E. ultrasonic disintegrator (peak-to-peak amplitude of 10 μm) for either four 15 s periods (samples 0-16 h) or for 12 15s periods (samples 16-20 h) with continuous cooling in an ice-salt bath to prevent excessive heating. The DNA content of sonicated cells prepared in this fashion was then determined by a modification of the procedure of Giles & Myers (1965): cold 50% (w/v) trichloroacetic acid (1 ml) was added to 4 ml of extract, and the mixture was incubated for 1 h at 0°C. The resulting precipitate was centrifuged (1000g, 20 min). The supernatant was discarded and the pellet was washed with two 2 ml portions of cold 10% (w/v) trichloroacetic acid. The pellet was resuspended in 2.5 ml of 5% (w/v) trichloroacetic acid and heated at 90°C for 30 min to hydrolyse the DNA. The hydrolysate was centrifuged (1000 g, 20 min) and to 1.6 ml of the clear supernatant was added 0.4 ml of 50% (w/v) $HClO_4$, 2.0 ml of 4% (w/v) diphenylamine in acetic acid and 0.2 ml of aqueous acetaldehyde (1.6 mg/ml). These reaction

mixtures were then incubated at 30°C for at least 20 h and the difference in extinction at 595 and 700 nm was determined. The amount of DNA was estimated by comparison with solutions of known deoxyribose content that had been treated identically.

Determination of Cellular Glycogen content

Cell extracts were prepared as described above. A suspension of the extract (2 ml) and 1 ml of 90% (w/v) KOH were heated at 100°C for 20 min. Insoluble material was removed by centrifugation at 1700 g for 10 min and extracted with 1 ml of 30% (w/v) KOH at 100°C for a further 20 min. After recentrifugation the two supernatants were combined and 0.2 ml of saturated Na_2SO_4 and 8 ml of ethanol were added. Incubation at 0°C for 10 min precipitated all the glycogen, which was collected by centrifugation at 38,000 g for 15 min. The pellet was dissolved in 3 ml of water and the glycogen was repreciptated by addition of 6 ml of ethanol. Repetition of this precipitation procedure gave a white solid, which was finally dissolved in 2 ml of 0.5 M H_2SO_4. This solution was heated at 100°C for 5 h to hydrolyse the glycogen and the pH was adjusted to 5-6 by repeated extractions with equal volumes of 10% (v/v) NN-dioctylmethylamine in chloroform. The glucose concentration in this hydrolysate was then measured enzymically either by the glucose oxidase method of Dahlquist (1961) or by the hexokinase procedure of England & Randle (1967). Both methods gave identical results. Glycogen concentrations are expressed as glucose equivalents after hydrolysis/10^8 cells.

Determination of Total Cellular Carbohydrate content

Total cellular carbohydrate content was determined as described in Chapter I.

The Growth and Development of [U-^{14}C]glucose and [U-^{14}C]aspartate labelled myxamoebae

Myxamoebae of D. discoideum strain Ax-2 were grown in 80 ml of axenic medium containing [U-^{14}C]glucose (25 μCi) and a final glucose concentration of 86 mM. Alternatively, the axenic medium contained [U-^{14}C]aspartate (3 μCi) either in the absence or the presence of added glucose to 86 mM final concentration.

Cultures were shaken in 250 ml Erlenmeyer flasks on a rotary shaker at 150-170 rev/min until the cell density approached 10^7 cells/ml (for cells in medium containing added glucose) and 6 x 10^6 cells/ml (for cells in medium lacking added glucose). The myxamoebae were harvested and allowed to develop at a density of 7.5 x 10^6 myxamoebae per 27 mm diameter Millipore filter support, as described by Sussman (1966), in the centre well of a Conway dish closed by a well-greased glass ground lid. The outer well contained 1 ml of 1 N sodium hydroxide to trap any ^{14}C-carbon dioxide released. Control dishes contained Millipore filter supports without cells.

Analysis of [U-^{14}C]glucose and [U-^{14}C]aspartate-labelled myxamoebae

Cell-free extracts of myxamoebae were obtained as described previously. 1.0 ml samples of these extracts were added to 10 ml of Triton-toluene scintillation fluid (Fox, 1968) for the determination

of total cellular radioactivity. Glycogen was isolated from cell-free extracts by the method of Cooper & Kornberg (1967), dissolved in 4 ml of water, and samples of this added to 10 ml of Triton-toluene scintillation fluid. Protein was isolated from cell-free extracts by addition of 1 ml 30%(w/v) TCA to 2 ml of extract and collection of the precipitated protein by centrifugation after 30 min incubation at 0°C. The crude protein precipitate was dissolved in 1 ml NaOH and insoluble material removed by centrifugation. This material was re-extracted with 1 ml of 1N NaOH and the alkaline extracts pooled and incubated at 0°C for 30 min with 1 ml of 80% (w/v) TCA to reprecipitate protein. This process was repeated twice more and the final, purified, protein precipitate dissolved in 1 ml of 1N NaOH. Samples of this were taken for determination of the radioactive content by the methods of Harlan (1963) and of the protein content as described by Lowry et al. (1951).

Mature fruiting bodies were harvested in water, sonicated as described above, and samples counted in scintillation fluid as described by Fox (1968). Other samples of this cell extract were used to determine glycogen and protein specific radioactivities as described above.

The PDF was also collected, by squeezing the Millipore support pads using protective gloves, centrifuged to remove cellulose fibres (1000 g, 10 min) and samples counted for radioactivity by the method of Fox (1968).

The 1N NaOH in the outer well of the Conway dishes was collected, the volume determined by using a graduated centrifuge tube

and samples were taken for determination of radioactivity (Harlan, 1963).

The specific radioactivity of the end-product saccharides, trehalose and cellulose was determined as described below.

Determination of Specific Radioactivity of Trehalose and Glucose

Cells were harvested at various times during the developmental phase and ultrasonicated as described previously. 4.5 ml of extract was added to 0.25 ml of 60% perchloric acid and the mixture incubated for 30 min at 0°C. The precipitated protein was removed by centrifugation (1000 g, 20 min), the supernatant neutralised using KOH, and the resulting precipitate of potassium perchlorate also removed by centrifugation. 0.25 ml saturated sodium sulphate and 10 ml absolute alcohol were added to 5 ml of neutralised perchloric acid extract, prepared as described above, and the mixture incubated at 0°C for 15 min. The precipitated glycogen was removed by centrifugation (38,000 g, 15 min) and the supernatant deionised by passage through "Amberlite" Monobed MB3 resin. The resin was washed with an equal volume of water, and the pooled solutions carefully evaporated to dryness under vacuum at 50°C, and the residue resuspended in 5 ml water. This solution was further deionised by passage through Monobed MB3 resin which was washed as before. The pooled solutions were evaporated to dryness at 50°C under vacuum using a Rotary Evapomix (Büchler Instruments Ltd., New York), resuspended in 0.1 ml water and spotted onto Whatman No. 4 chromatography paper. The chromatogram was developed using isopropanol : n-butanol : water, (140 : 20 : 40)

and carbohydrates were detected using alkaline silver nitrate (Trevelyan et al., 1950) and sodium metaperiodate (Evans & Dethier, 1957), and identified by comparison with authentic samples run on the same paper. Trehalose and glucose were eluted according to the method of Dimler et al.(1952), concentrated at 50°C using a Rotary Evapomix and each redissolved in 2 ml of water. Samples were assayed for trehalose and glucose (as described below) and for radioactivity by the method of Fox (1968).

Determination of Trehalose

Trehalose was prepared from Neurospora crassa by a modification of the method of Hill & Sussman (1963) as described in the Appendix. The enzyme was active only on trehalose when tested on a large range of carbohydrates including glycogen, lactose, maltose, cellulose and cellobiose (see Appendix).

Each assay mixture for the assay of trehalose contained 0.8 ml of sample, pH 7.8, 0.1 ml of 0.5 M phosphate buffer, pH 5.6, and 0.1 ml of trehalase. This was incubated for 4 h at 37°C, boiled for 10 min to stop further reaction, and the glucose content of the solution determined using the hexokinase assay of England & Randle (1967). This value was corrected for the glucose content of pre-trehalase extract also determined by the hexokinase assay of England & Randle (1967).

Determination of Specific Radioactivity of Cell Wall Polysaccharide

Mature fruiting bodies (40 h developmental time) were harvested into water and sonicated as described previously. 2 ml of

cell extract was added to 1 ml 90% (w/v) KOH and the suspension heated at 100°C for 20 min. Insoluble material was removed by centrifugation at 17,000 g for 10 min and extracted with 1 ml 30% (w/v) KOH at 100°C for a further 20 min. After recentrifugation, the pellet was washed twice with 2 ml water each time. Some samples were filtered through 25 mm diameter Sartorius Membrane filters (cat. no. 11306), washed twice with 5 ml water, dried under infra red lamp, and counted for radioactivity in 10 ml toluene scintillation mixture. Other samples were dissolved in 67% (w/v) H_2SO_4 (2 h at room temp.) and assayed for hexose by the anthrone method of Hassid & Abraham (1957).

Measurements of radioactivity were carried out in a Packard Tricarb Liquid Scintillation Spectrometer model 43/2 at approx. 35-45% efficiency. Variations in quenching were allowed for using the channels ratio method of Baillie (1963).

Assay of Phosphorylase

Phosphorylase activity was assayed according to the method of Firtel & Bonner (1972) using cell extracts prepared in water and stored frozen at -35°C, except that assay mixtures contained 0.1 International Units of 6-phosphogluconate dehydrogenase and the assay was performed at 23°C.

Assay of Amylase

Amylase activity was assayed according to the method of Jones & Wright (1970) but with some modifications.

Reaction mixtures contained the following in a total volume of 0.3 ml: buffer [either 3.0 µmoles of sodium acetate, pH 4.8, or 30 µmoles of imidazole HCl, pH 6.9], 1.0 µmoles NaCl, 2.0 mg glycogen, and 0-0.2 ml cell extract (prepared as described previously). Control reactions containing cell extract but no glycogen were used to correct for reducing sugar present in the extract, and reaction mixtures containing maltose (0-1.2 µmoles) but no enzyme were used to prepare a calibration curve.

The reaction was allowed to proceed at 23°C for 60 min, and 0.2 ml of colour reagent (1% 3,5-dinitrosalicyclic acid - 30% potassium sodium tartrate in 0.4 N NaOH) was added. The reaction tubes were then heated at 100°C for 5 min, cooled, and 2.0 ml water added to each. Following centrifugation (1000 g, 10 min) to remove any insoluble material, the optical density of the supernatant was measured at 540 nm. The rate of production of reducing sugar was linear with respect to the amount of extract used or with the length of incubation up to at least 60 min.

Assay of Fructose 1,6-diphosphatase (FDPase)

Axenically-grown myxamoebae were allowed to develop, cell samples were collected at intervals, and extracts prepared as described previously, except that cells were harvested in 10 mM Tris, pH 7.0, containing 1 mM cysteine and 1 mM EDTA. Cell extracts were assayed for FDPase activity according to the method of Rose (1966).

Assay of Maltase

Axenically-grown myxamoebae were harvested and allowed to develop at 22°C on Millipore filters at a cell density of approx. 2.5×10^7 cells per filter, as described previously. At intervals during the developmental phase, the cells from 5 such filters were harvested into 3 ml of ice cold water and stored at -15°C. After thawing the cell suspensions slowly in the cold, cell extracts were prepared as described previously, and assayed for maltase activity as follows.

Assay mixtures contained 80 µmoles of sodium phosphate-citrate buffer, pH 4.0, 2 mg maltose, 0.1 ml cell extract, made to 1 ml final volume with water, and incubated at 23°C for 1 h. The reaction was terminated by boiling the assay mixture for 10 min and glucose assayed by the hexokinase method of England & Randle (1967). Control reactions containing extract but no maltose, and maltose but no extract, were used to correct for glucose present in cell extracts and maltose solution respectively.

RESULTS

Glycogen content of Cells during Development

The percentage recovery of cells from Millipore filters varies during the developmental sequence. For example, it is much easier to wash off the slug stages (14-16 h) by our harvesting procedures than it is to recover aggregating cells (0-7 h). To correct for this inherent variability, the results have been normalised to glycogen contents/10^8 cells by measuring in the same sample the amount of DNA and assuming that:

(i) the DNA content of 10^8 myxamoebae is 17.86 ± 0.30 μg (24)*

(ii) the DNA content per cell does not change during development [Leach & Ashworth (1972) have shown that the DNA content of spore cells and vegetative myxamoebae are approximately equivalent.]

Fig. 5 shows the recoveries obtained, in terms of sample DNA content, in a typical series of experiments.

Myxamoebae grown in the absence of added glucose contain very low levels of glycogen (often less than 0.1 mg per 10^8 cells) and this is rapidly degraded during the first few hours of development (Fig. 6). Glycogen is then synthesised during the late aggregation and slug migration stages (5-15 h) and finally broken down at the time of end-product saccharide synthesis. The amount of glycogen

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* The number in brackets refers to the number of determinations. Each determination is the average of three measurements on a separate cell culture.

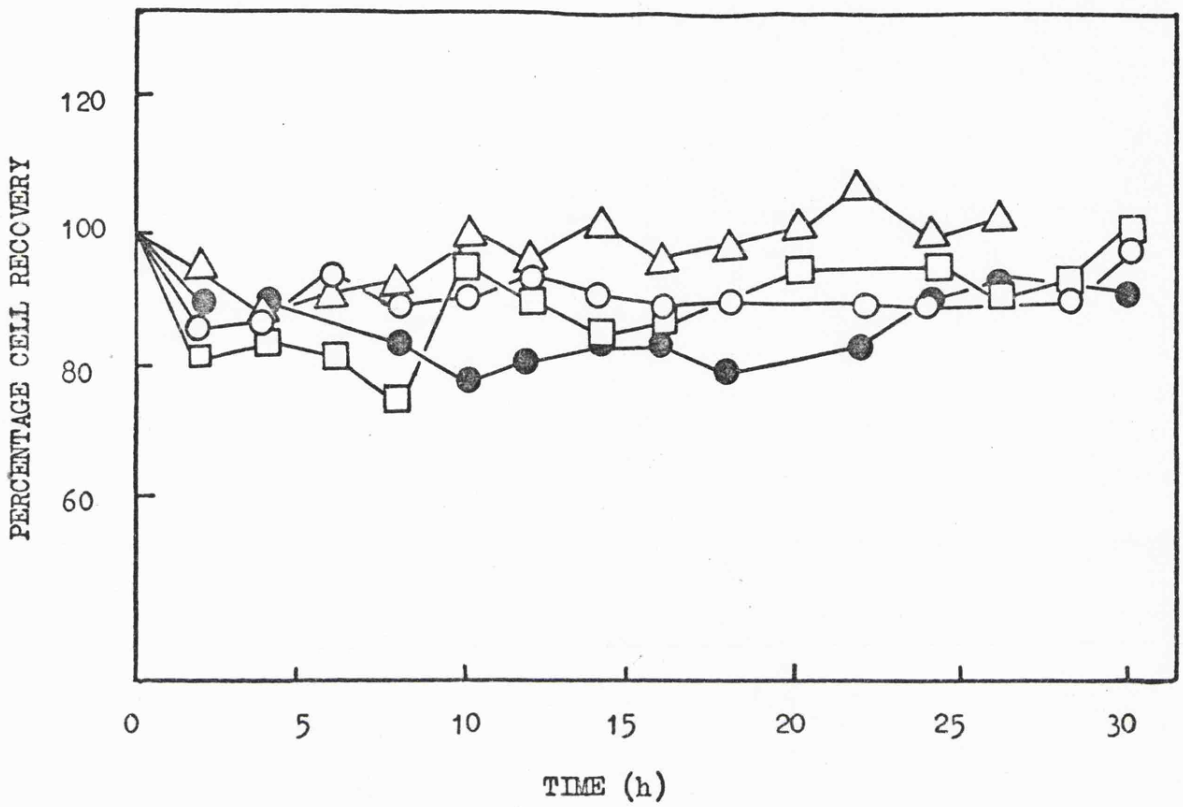

FIG.5 Cell Recovery during The Development of D.discoideum Ax-2 Myxamoebae. Cells were harvested from Millipore filters at the times indicated and the DNA content of the cell sample determined. The percentage of cells recovered was then calculated for each cell sample assuming that:-

(1) the DNA content of 10^8 myxamoebae of D.discoideum strain Ax-2 is 17·86μg (see RESULTS)

(2) the DNA content per cell does not change during development (Leach @ Ashworth 1972)

Several typical experiments are shown.

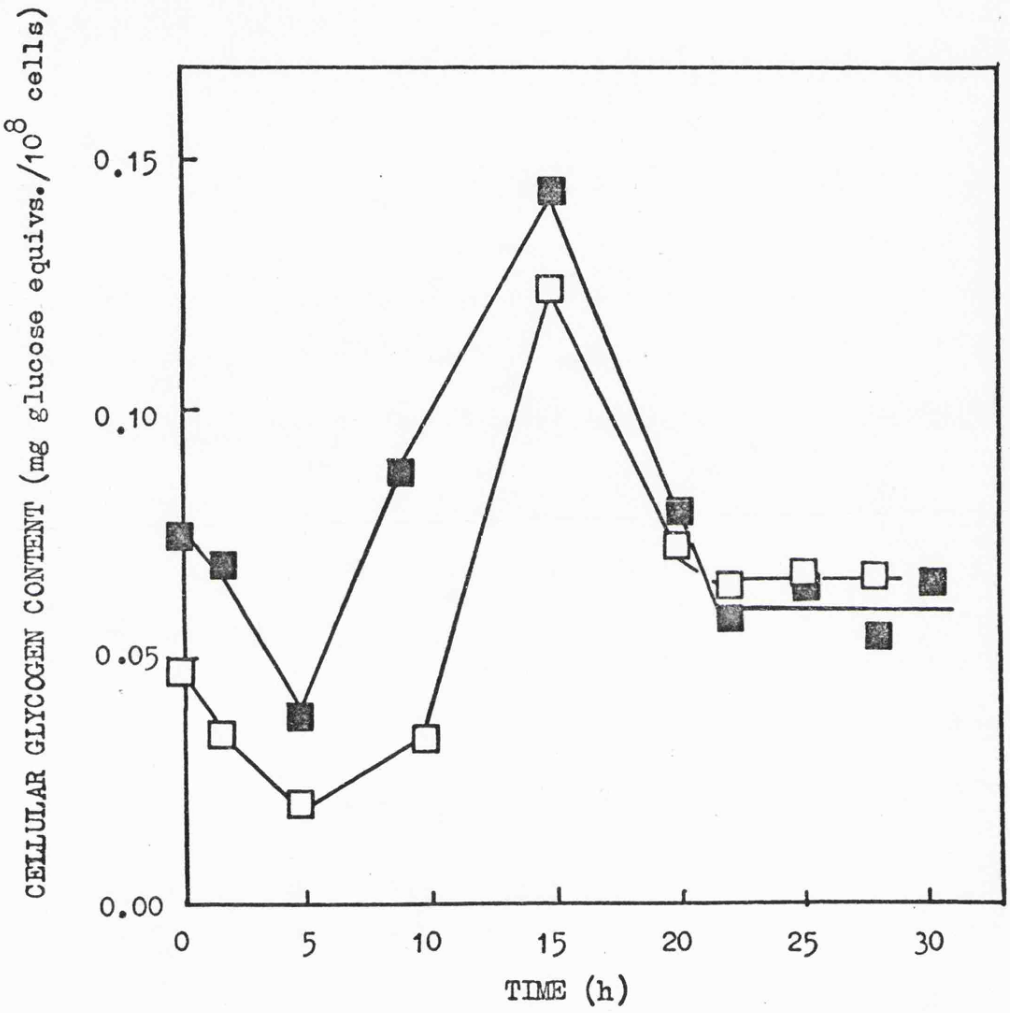

FIG. 6 Changes in The Glycogen Content of Axenically-grown Cells during Development.

□ ; myxamoebae initially containing 0·046mg glycogen/10^8 cells.

■ ; myxamoebae initially containing 0·074mg glycogen/10^8 cells.

synthesised during this time is considerably greater than the vegetative myxamoebal glycogen content and synthesis occurs when the cellular pool of glucose remains at a constant, low, level (Fig. 7), suggesting that gluconeogenesis is occurring. This is confirmed by the finding that the total cellular carbohydrate content, following an initial decrease presumably due to myxamoebal glycogen degradation, doubles during the developmental stage (Fig. 8).

Myxamoebae initially containing more than 1 mg glycogen per 10^8 cells also exhibit glycogen degradation during development but the glycogen degradation phase now persists throughout development and net synthesis of glycogen is never observed (Fig. 9). For myxamoebae initially containing up to approximately 4 mg glycogen per 10^8 cells, the rate of glycogen degradation increases with increasing myxamoebal glycogen content so that the mature fruiting bodies all contain 0.083 ± 0.026 (4)* mg glycogen per 10^8 cells. However, myxamoebae containing more than 5 mg glycogen per 10^8 cells appear to be unable to degrade the glycogen at a fast enough rate to achieve this low fruiting body glycogen content, such that the fruiting bodies formed contain much higher levels of glycogen (approximately 0.5 mg glycogen/ 10^8 cells) (Fig. 9).

Fate of Degraded Glycogen

Myxamoebae initially containing more than 1 mg glycogen/10^8 cells exhibit a large loss of total cellular carbohydrate during

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* As footnote on p. 26

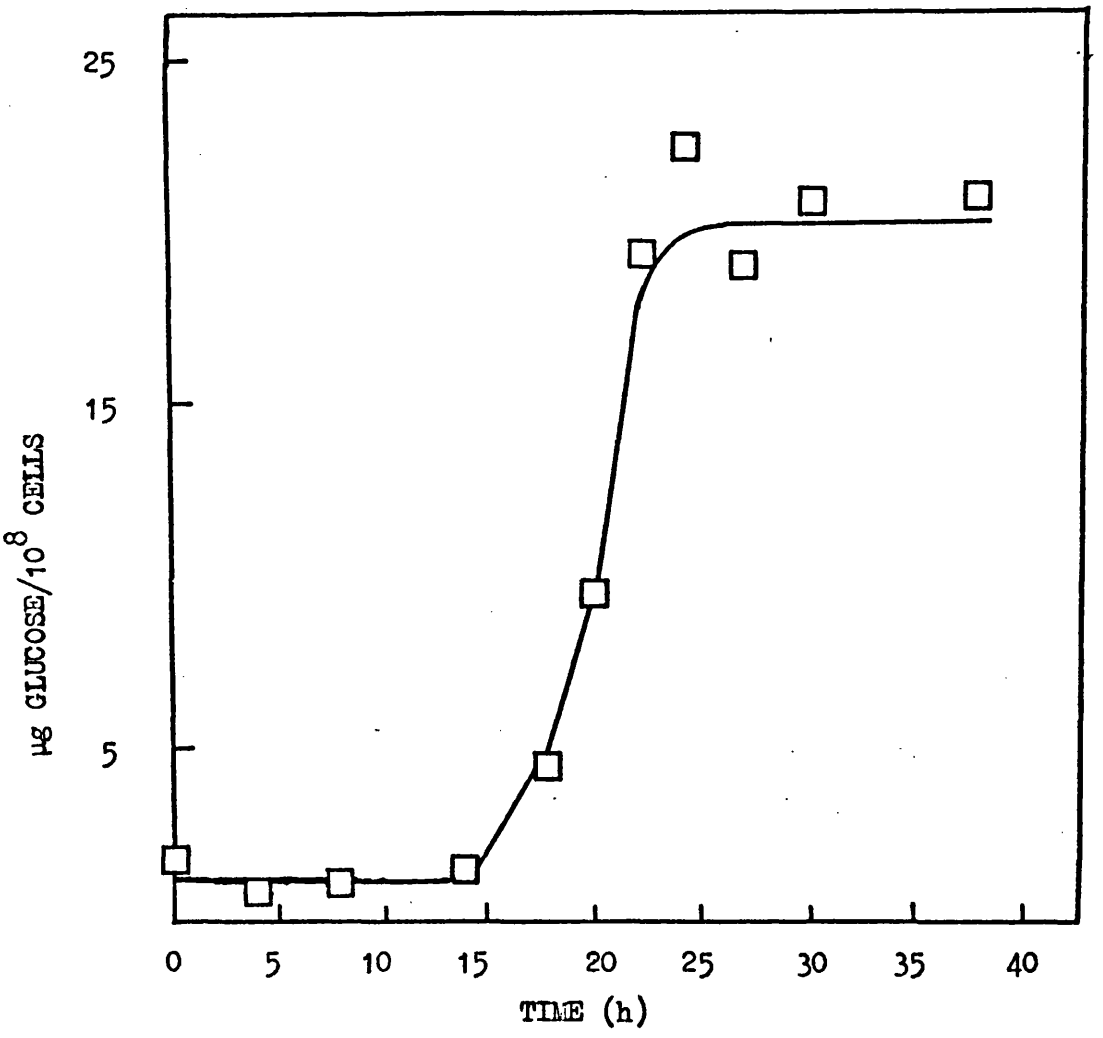

FIG. 7 Changes in The Cellular Content of Glucose during The Development of Axenically-grown Myxamoebae.

The myxamoebae initially contained 0·13mg glycogen/10^8 cells.

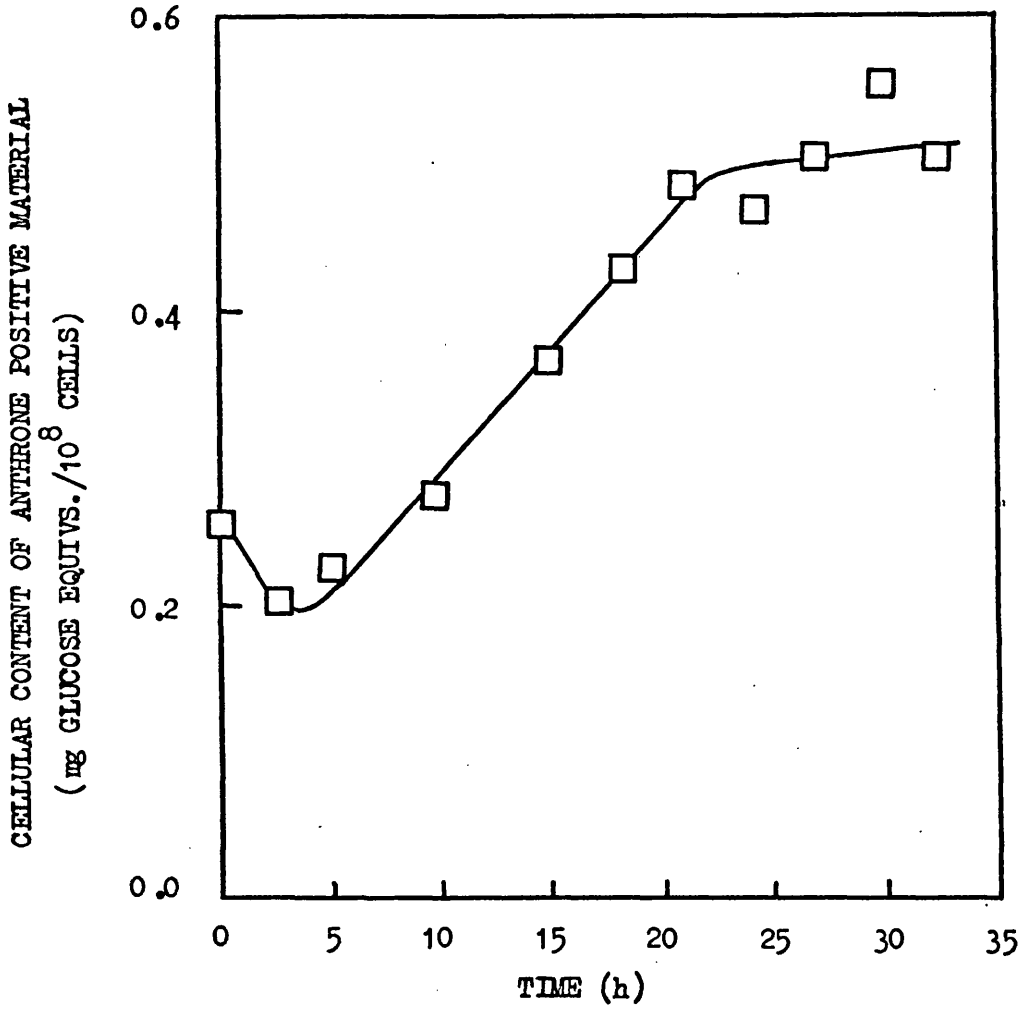

FIG. 8 Changes in The Cellular Content of Anthrone Positive Material during The Development of Axenically-grown Myxamoebae.
The myxamoebae initially contained 0·074mg glycogen/10^8 cells.

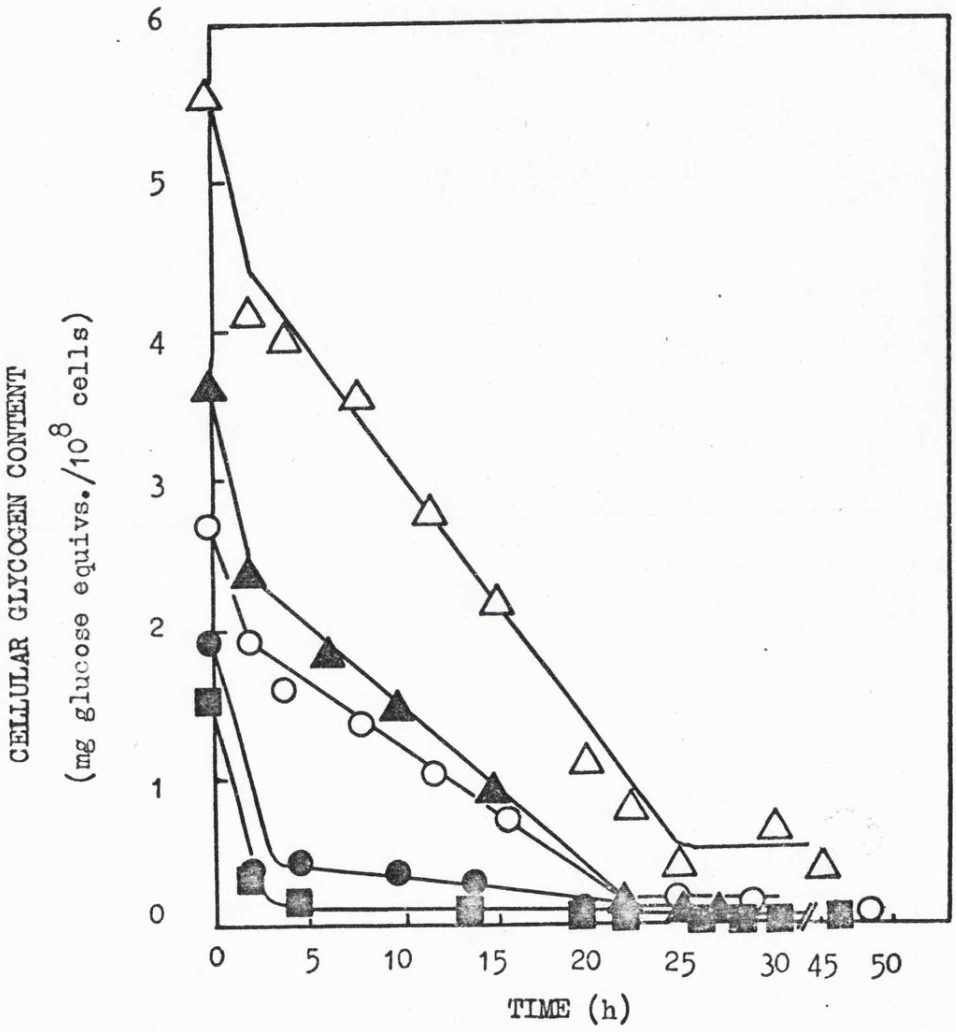

FIG. 9 Changes in The Cellular Content of Glycogen during The Development of Axenically-grown Myxamoebae.

Myxamoebae initially contained, per 10^8 cells;

■ ; 1·49mg glycogen

● ; 1·98mg glycogen

○ ; 2·67mg glycogen

▲ ; 3·62mg glycogen

△ ; 5·56mg glycogen

development (Fig. 10). Since at least 90% of the anthrone-positive material of myxamoebae can be accounted for by their glycogen content (Ashworth & Watts, 1970; Chapter I), this suggests that the major part of myxamoebal glycogen is metabolised to non-hexose material(s). Indeed, studies with myxamoebae containing uniformly labelled glycogen, as a consequence of their growth in [U-^{14}C]glucose-containing medium, have shown that the major product of myxamoebal glycogen metabolism is CO_2 (Table 2a, b)*. Measurements of the radioactivity of the PDF and cell wash water indicate that no significant cellular excretion of glycogen degradation products occurs during development (Table 2a, b).

However, apparently not all the myxamoebal glycogen is oxidised to carbon dioxide since comparison of the glycogen and total carbohydrate contents of mature sorocarps indicates that myxamoebae initially containing high levels of glycogen produce fruiting bodies which contain more non-glycogen carbohydrate than those produced by myxamoebae initially containing low glycogen contents (Table 3). This raises the possibility, investigated in detail in Chapter III, that some myxamoebal glycogen is used to synthesise increased levels of end-product saccharides.

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* I realise that the growth of cells in [U-^{14}C]glucose-containing medium will label cellular components other than glycogen and protein which may be oxidised to carbon dioxide during development (e.g. ribose of RNA) but the data in Table 2b indicate that most of the cellular radioactivity loss during development is due to glycogen degradation and that almost all the radioactivity lost can be trapped as carbon dioxide. That the gas was indeed carbon dioxide was shown by mass spectrometry as described in Appendix B.

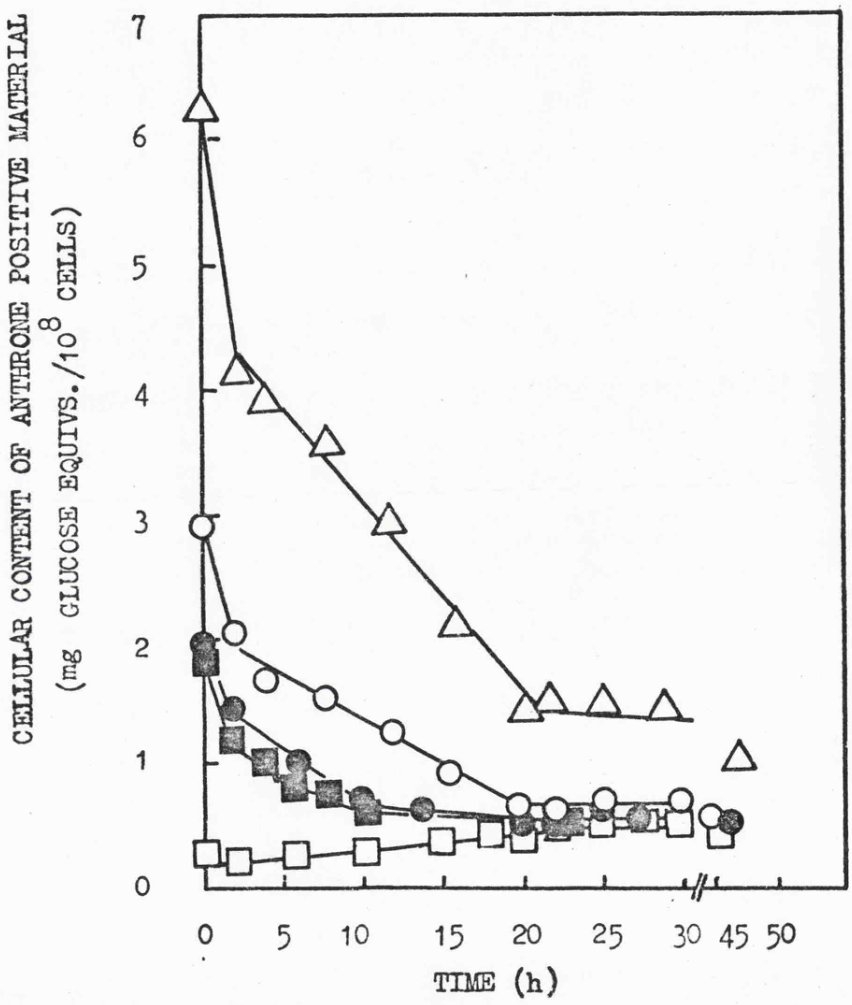

FIG. 10 Changes in The Cellular Content of Anthrone Positive Material during The Development of Axenically-grown Myxamoebae.

Myxamoebae initially contained, per 10^8 cells;

□ ; 0·074mg glycogen

■ ; 1·49mg glycogen

● ; 1·98mg glycogen

○ ; 2·67mg glycogen

△ ; 5·56mg glycogen

TABLE 2 (a) The oxidation of myxamoebal glycogen to carbon dioxide during development

Sample (material and development time)		Total Radio-activity (cpm/10^8 cells	Cellular content of material (μmoles/10^8 cells)	Specific Radioactivity (cpm/μg atom carbon)
Total cells	0 h	551,357	-	-
	150 h	139,239	-	-
Cellular glycogen				
	0 h	398,000	34.78	1,907
	33 h	63,017	5.75	1,825
	150 h	23,072	1.86	2,071
Cellular protein				
	0 h	123,500*	101.50 *	243
	33 h	51,200*	35.80 *	286
	150 h	35,500*	28.00 *	253
Carbon dioxide	150 h	369,056	-	-
PDF	150 h	12,083	-	-
Sorocarp wash water	150 h	Counts little above background		

Myxamoebae used in this experiment initially contained 6.26 mg glycogen/10^8 cells. All radioactivities are corrected for quenching as described in the text.

* These figures are estimates based on:

(1) the specific radioactivity of cellular TCA insoluble protein
(2) the total protein content of cell extracts, and thus do not take account of the specific radioactivity of cellular TCA soluble protein
(3) the assumption that the average molecular weight of the amino acids of D. discoideum protein is 100.

TABLE 2 (b) The oxidation of myxamoebal glycogen to carbon dioxide during development

Sample	Total Radioactivity lost or gained during the 0-150 h period of development ($cpm/10^8$ cells)	Total Radioactivity lost or gained as a percentage of the total cell radioactivity lost during the 0-150 h period of development
LOSS		
Total cells	442,118	100.00
Cellular glycogen	374,938	85.00
Cellular protein	88,000*	19.90
GAIN		
Carbon dioxide	369,056	83.50
PDF	12,083	2.73
Sorocarp wash water	negligible	-

* This figure is an estimate (see Table 2(a))

TABLE 3 The relationship between myxamoebal glycogen content and the sorocarp content of non-glycogen anthrone positive material

Myxamoebal glycogen content ($mg/10^8$ cells)	Sorocarp content of non-glycogen anthrone positive material (μg glucose equivs./10^8 cells)
0.046 (1)	0.483 (1)
1.623 ± 0.179 (3)	0.576 ± 0.029 (3)
2.67 (1)	0.660 (1)
5.56 (1)	1.000 (1)

Results are given as means $\pm$ S.E.M.

Figures in brackets refer to the number of experiments

Myxamoebal Glycogen and "Developmental" Glycogen

The data described above indicate that myxamoebal glycogen is rapidly degraded during development, irrespective of myxamoebal glycogen content, to carbon dioxide. However, the studies with myxamoebae initially containing less than 0.1 mg glycogen/10^8 cells showed that during development, glycogen was synthesised and only broken down at the time of end-product saccharide synthesis. This suggests that possibly two pools of glycogen exist in developing cells; one, a pool of myxamoebal glycogen oxidised during development to carbon dioxide, and another pool of "developmental" glycogen, synthesised during development, for use as precursor for end-product saccharide synthesis. The synthesis of developmental glycogen could occur unobserved in myxamoebae containing more than 1 mg glycogen per 10^8 cells provided that the developmental glycogen pool remained small in relation to the myxamoebal glycogen pool. The synthesis of developmental glycogen would then be masked by the degradation of a larger myxamoebal glycogen pool.

The following studies were made in order to test this two-pool hypothesis:

1. Rate of glycogen synthesis during development If a developmental pool of glycogen is synthesised during the developmental phase of myxamoebae containing high levels of myxamoebal glycogen, then it should be detectable by isotopic measurement of the rate of glycogen synthesis.

Weeks (in Hames et al., 1972) measured the absolute rate of degradation of ^{14}C-glycogen in developing cells which initially

contained 2.63 mg [U-^{14}C]glycogen per 10^8 myxamoebae and, by comparison of this rate with the net rate of degradation at various times during the developmental phase, was able to determine the rate of glycogen synthesis (Fig. 11). It is clear that the rate of glycogen synthesis during the development of myxamoebae containing high levels of glycogen parallels the synthesis of developmental glycogen during the development of myxamoebae containing less than 0.1 mg glycogen per 10^8 cells (Fig. 6).

2. Isotopic labelling of glycogen pools The developmental pool of glycogen synthesised during the development of myxamoebae containing less than 0.1 mg glycogen per 10^8 cells is synthesised using hexose units derived from gluconeogenesis (see above). If a similar pool is synthesised during the development of myxamoebae initially containing high glycogen levels, it should be detectable by specific pool labelling experiments.

Myxamoebae were grown in [U-^{14}C]glucose-containing medium to uniformly label their myxamoebal glycogen, and allowed to develop. The fruiting bodies were harvested, cellulose, trehalose and glucose isolated and their specific radioactivities determined (Table 4). The data show that even though myxamoebal glycogen is degraded and radioactivity from this pool finds its way into end-product saccharides, the specific radioactivity of the latter is approximately 50-70% that of myxamoebal glycogen. Thus a non-myxamoebal glycogen source of carbon is used to synthesise at least some of the end-product saccharides.

Attempts were made to demonstrate the synthesis of developmental

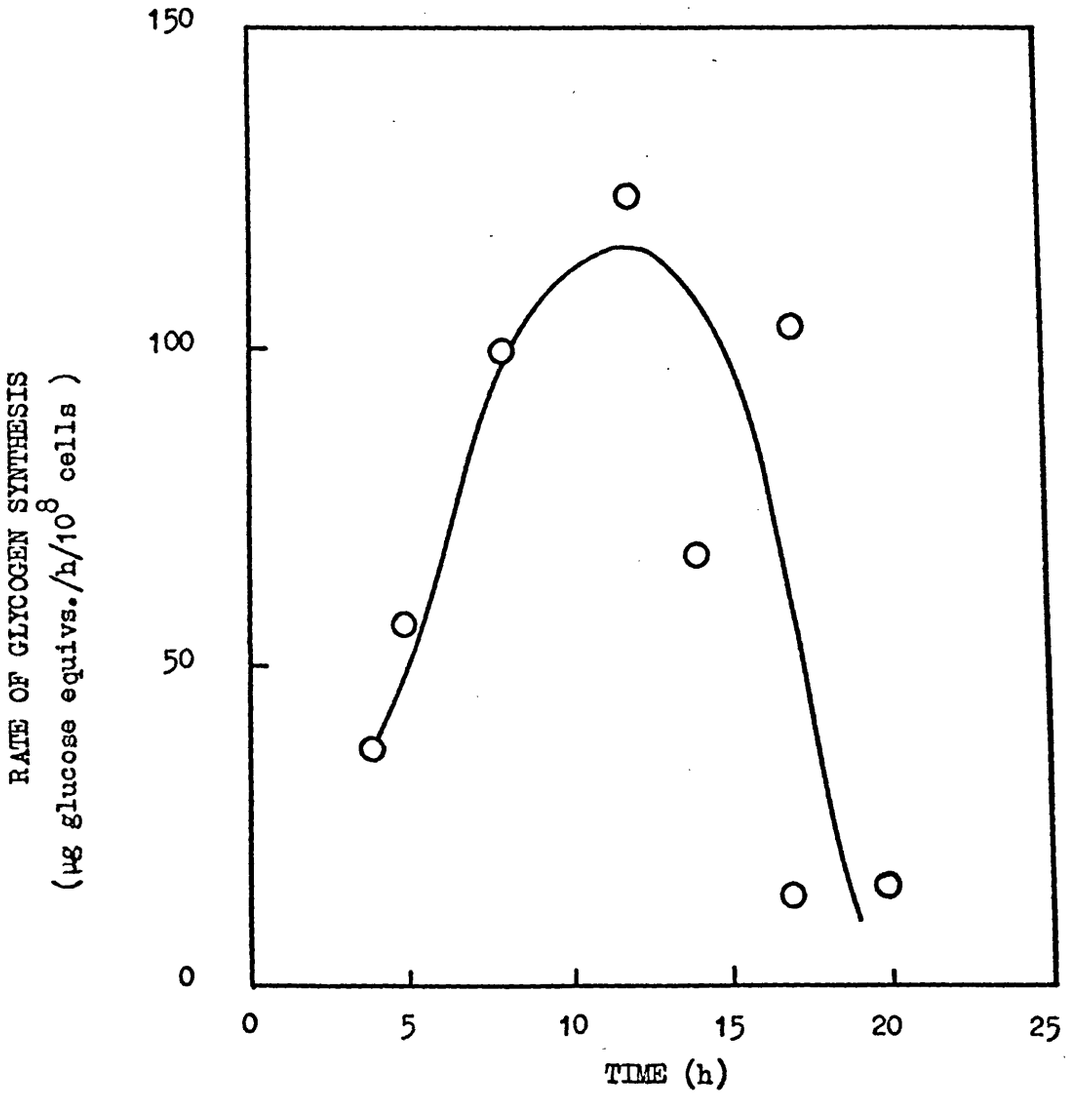

FIG. 11 Variation in The Rate of Glycogen Synthesis durind Development of Myxamoebae that initially contained 2·63mg Glycogen/10^3 Cells.

(Figure taken from Hames, Weeks @ Ashworth 1972)

TABLE 4 The use of myxamoebal glycogen as precursor for end-product saccharide synthesis

Compound	Specific Radioactivity (cpm/μg atom carbon)	
	Experiment I	Experiment II
Cellular glycogen		
0 h	1,820	1,806
40 h	-	1,910
Cellular protein		
0 h	188	257
40 h	-	274
Cell wall polysaccharide	1,120	1,110
Trehalose	-	927
Glucose	-	1,251

Experiment I : Myxamoebae initially contained 6.10 mg glycogen/10^8 cells

Experiment II: Myxamoebae initially contained 5.25 mg glycogen/10^8 cells

glycogen, during the development of myxamoebae initially containing high levels of glycogen, by specific labelling of myxamoebal protein as a gluconeogenic substrate. However, myxamoebae grown in axenic medium containing added glucose and [U-^{14}C] aspartate incorporate label into myxamoebal glycogen almost as well as into myxamoebal protein, making such an experiment impractical.

3. Phosphorylase and amylase activities during development. During the early stages of development, axenically-grown myxamoebae possess high levels of amylase activity (Fig. 12) and, since cellular glycogen phosphorylase activity is extremely low at this time (Fig. 13, Table 5), myxamoebal glycogen degradation must be due to amylytic action. Although no detailed studies of the products of digestion have been undertaken, the fact that the amylase activity of D. discoideum is optimal at acid pH (Fig. 12) suggests that it is likely to be a β-amylase (Bernfeld, 1955) degrading glycogen to maltose, which would be further hydrolysed to glucose by cellular maltase (Table 7).

However, even though cellular amylase and maltase activities are capable of degrading large amounts of myxamoebal glycogen, phosphorylase activity increases dramatically during fruiting body construction* (Fig. 13).

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*The apparent peak of amylase activity observed at 20h developmental time (Fig 12) is probably due to this phosphorylase activity since whereas amylase activity is greatly reduced at pH 7.0, the 20h peak activity is not (Fig. 12). Phosphorylase activity would be detected by the assay for amylase since axenically-grown myxamoebae possess a phosphatase activity throughout development which reacts with the G1P product of phosphorylase action (Table 6).

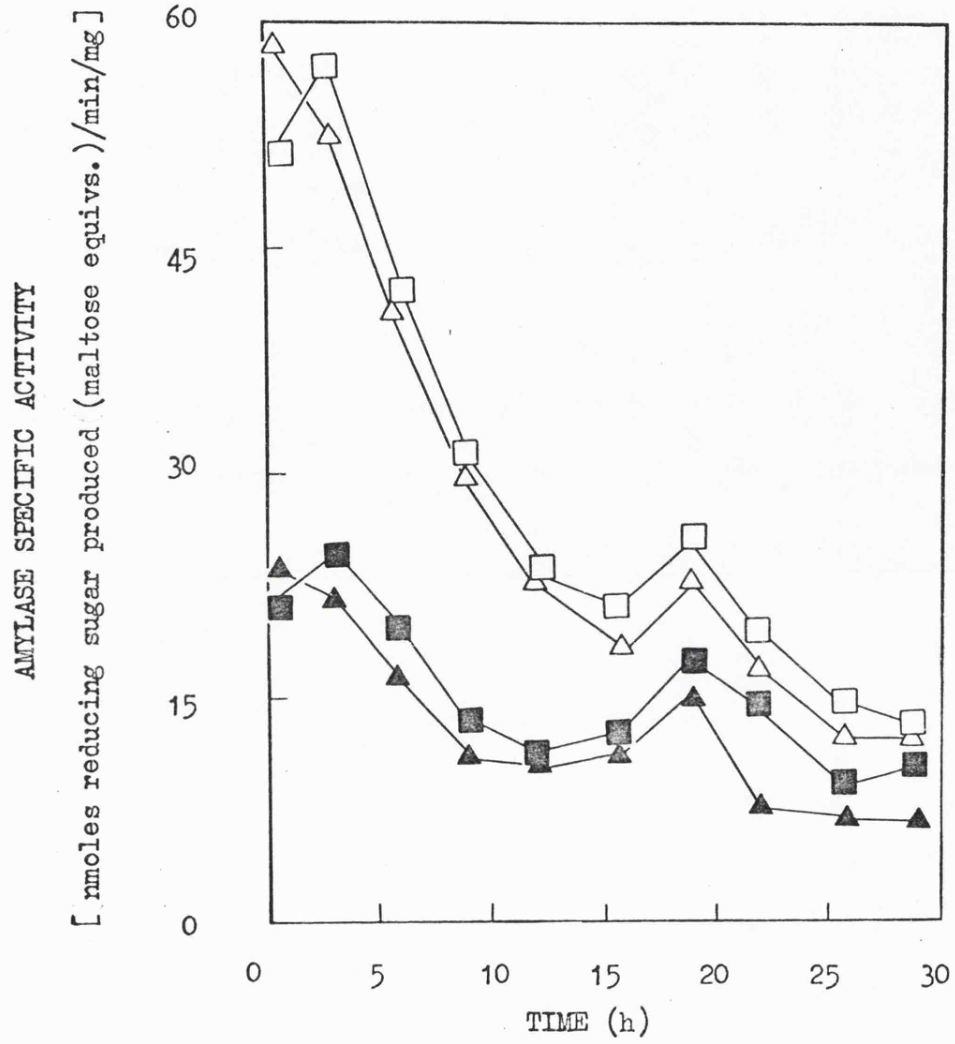


FIG. 12 Variation in Amylase Activity during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially containing 0·27mg glycogen/10^8 cells and assayed for amylase activity at pH 4·8

△ ; myxamoebae initially containing 7·50mg glycogen/10^8 cells and assayed for amylase activity at pH 4·8

■ ; myxamoebae initially containing 0·27mg glycogen/10^8 cells and assayed for amylase activity at pH 6·9

▲ ; myxamoebae initially containing 7·50mg glycogen/10^8 cells and assayed for amylase activity at pH 6·9

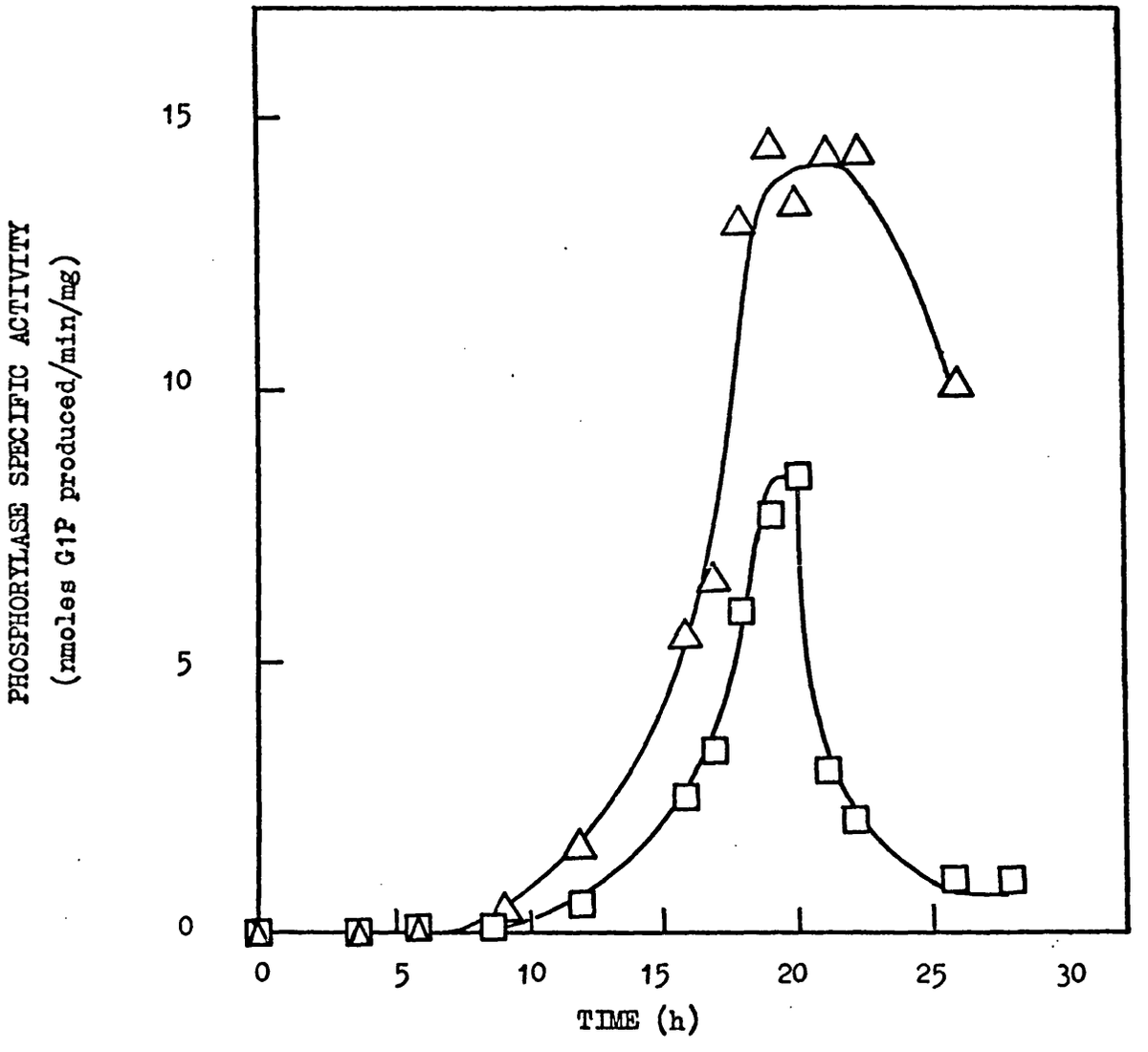


FIG. 13 Variation in Glycogen Phosphorylase Activity during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially containing 0·30mg glycogen/10^8 cells

△ ; myxamoebae initially containing 5·35mg glycogen/10^8 cells

TABLE 5 The cellular activity of glycogen phosphorylase prior to aggregation of axenically-grown myxamoebae

Developmental time (h)	Glycogen phosphorylase specific activity (nmoles G1P produced/min/mg)	
	NS*	G*
0	0.046 ± 0.023 (3)	0.060 (1)
3	0.090 ± 0.013 (3)	0.087 (1)
6	0.103 ± 0.024 (3)	0.063 (1)

* NS: Myxamoebae initially contained 0.47 ± 0.18 (3) mg glycogen/10^8 cells

* G : Myxamoebae initially contained 8.50 (1) mg glycogen/10^8 cells

Results are given as means ± S.E.M.

Figures in brackets refer to the number of experiments

TABLE 6 The glucose 1-phosphatase activity of axenically-grown myxamoebae during the developmental phase

Developmental time (h)	Amylase activity [nmoles reducing sugar (maltose equivs.)/min/mg]		Plus G1P x 100% / Minus G1P
	*Minus G1P	*Plus G1P	
**NS			
0	21.62	27.20	126
11	11.30	16.25	144
22	6.55	9.75	149
** G			
0	22.50	25.30	113
11	8.30	11.50	139
22	4.25	6.32	149

* Minus G1P: cell extracts were assayed for amylase activity according to the method described in the text

* Plus G1P : cell extracts were assayed for amylase activity as described in the text except that reaction mixtures also contained 2 mg G1P

** NS: myxamoebae initially contained 0.29 mg glycogen/10^8 cells

** G: myxamoebae initially contained 5.85 mg glycogen/10^8 cells

TABLE 7 The maltase activity of axenically-grown cells during the developmental phase

Developmental time (h)	Cellular maltase activity (nmoles glucose produced/min/mg)
*NS	
0	20.50
11	9.55
22	7.20
*G	
0	19.38
11	6.34
22	4.48

*NS: myxamoebae initially containing 0.29 mg glycogen/10^8 cells

*G : myxamoebae initially containing 5.85 mg glycogen/10^8 cells

Note: maltase assays were carried out at pH 4.0 since this is the pH optimum for D. discoideum maltase activity (Fig. 14).

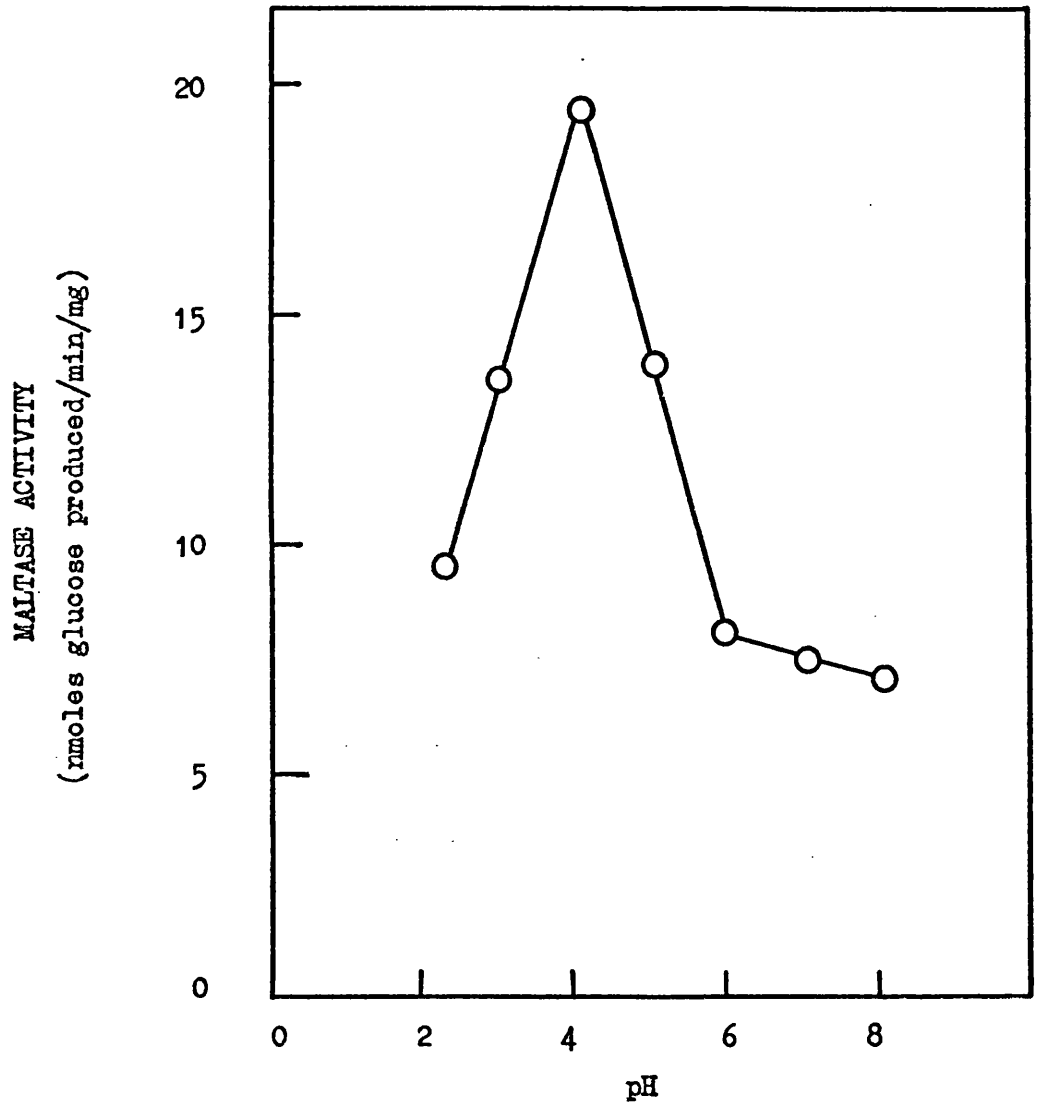


FIG. 14 The Influence of pH on The Activity of Maltase in Cell Extracts of D.discoideum.

Sodium phosphate-citrate buffer was used throughout this study as described in METHODS.

For what reason? One is tempted to suggest that amylase activity is active only on myxamoebal glycogen and that degradation of developmental glycogen during fruiting body construction requires the synthesis of glycogen phosphorylase. This will be discussed in more detail later in this thesis.

Bacterially-grown myxamoebae have also been reported to synthesise glycogen phosphorylase during development (Jones & Wright, 1970; Firtel & Bonner, 1972), in a manner similar to that described above for axenically-grown cells. However, bacterially-grown myxamoebae are reported to lack phosphorylase activity completely prior to aggregation (Firtel & Bonner, 1972), whereas this is not so of axenically-grown cells (Table 5).

There is one further interesting observation concerning phosphorylase accumulation by developing cells of D. discoideum; during the development of axenically-grown myxamoebae, the peak specific activity of phosphorylase appears to be a function of the myxamoebal glycogen content such that myxamoebae initially containing 6.87 ± 0.91 mg glycogen/10^8 cells synthesise twice the amount of phosphorylase as myxamoebae initially containing 0.32 ± 0.06 mg glycogen/10^8 cells (Fig. 13, Table 8).

That the difference in phosphorylase activity between these two sets of cells is not due to differences in the intracellular levels of enzyme activators or inhibitors is shown by the fact that the activities of mixtures of cell extracts were the sums of the activities

displayed by the separate extracts (Table 9). This, of course, does not give any information about the presence of inhibitors or activators which may be bound to enzyme molecules and not in excess.

TABLE 8 The dependence of peak glycogen phosphorylase specific activity attained during development upon myxamoebal glycogen content

Myxamoebal glycogen content ($mg/10^8$ cells)	Peak glycogen phosphorylase specific activity during development (nmoles G1P produced/min/mg)
0.32 ± 0.06 (5)	6.62 ± 0.060 (5)
0.70 ± 0.13 (2)	6.79 ± 1.61 (2)
6.87 ± 0.91 (3)	12.35 ± 1.16 (3)

Results are given as means $\pm$ S.E.M.

Figures in brackets refer to the number of experiments

TABLE 9 To show the absence of excess amounts of activators or inhibitors of glycogen phosphorylase in cell extracts of axenically-grown myxamoebae during the developmental phase

*Sample	Measured Phosphorylase activity (ΔOD_{340}/min)	Expected Phosphorylase activity (ΔOD_{340}/min)
NS 16	0.330	-
G 18	0.700	-
NS 16 + G 18	0.950	1.030
NS 19	0.240	-
G 20	0.690	-
NS 19 + G 20	1.000	0.930

* Each sample is denoted in terms of initial myxamoebal glycogen content (NS or G - see below) and the developmental time of sampling (in h)

NS: myxamoebae initially containing 0.25 mg glycogen/10^8 cells

G : myxamoebae initially containing 6.75 mg glycogen/10^8 cells

DISCUSSION

A Wright's Model of Control of Carbohydrate Metabolism during Development

The enormous interest in recent years in the carbohydrate metabolism of D. discoideum during the developmental phase stems from the discovery of White & Sussman (1961) that both qualitative and quantitative changes occur in the carbohydrate content of developing cells which directly reflect the morphological events of development. This, and subsequent work, showed that, during development, cellular alkali soluble glycogen initially decreased in concentration was later resynthesised and finally degraded during fruiting body construction (Fig. 15B) at a time when an alkali insoluble cellulose-glycogen cell wall complex (White & Sussman, 1961; Ward & Wright, 1965), an acid mucopolysaccharide (White & Sussman, 1963b), and trehalose (Ceccarini & Filosa, 1965) were being synthesised as "end-products" of differentiation. However, Wright & Dahlberg (1967), in contrast to the results of White & Sussman (1961), reported that cellular glycogen (measured as the glycogen content of the pellet sedimenting at 100,000 g) was conserved during development and only degraded during fruiting body construction (Fig. 15A). Wright et al. (1968b) thus presented a "kinetic model of metabolism essential to differentiation in Dictyostelium discoideum" in which glycogen synthesis and breakdown are key control events.

The Model

The model assumes that the pattern of carbohydrate metabolism during the development of myxamoebae of D. discoideum may be depicted as:

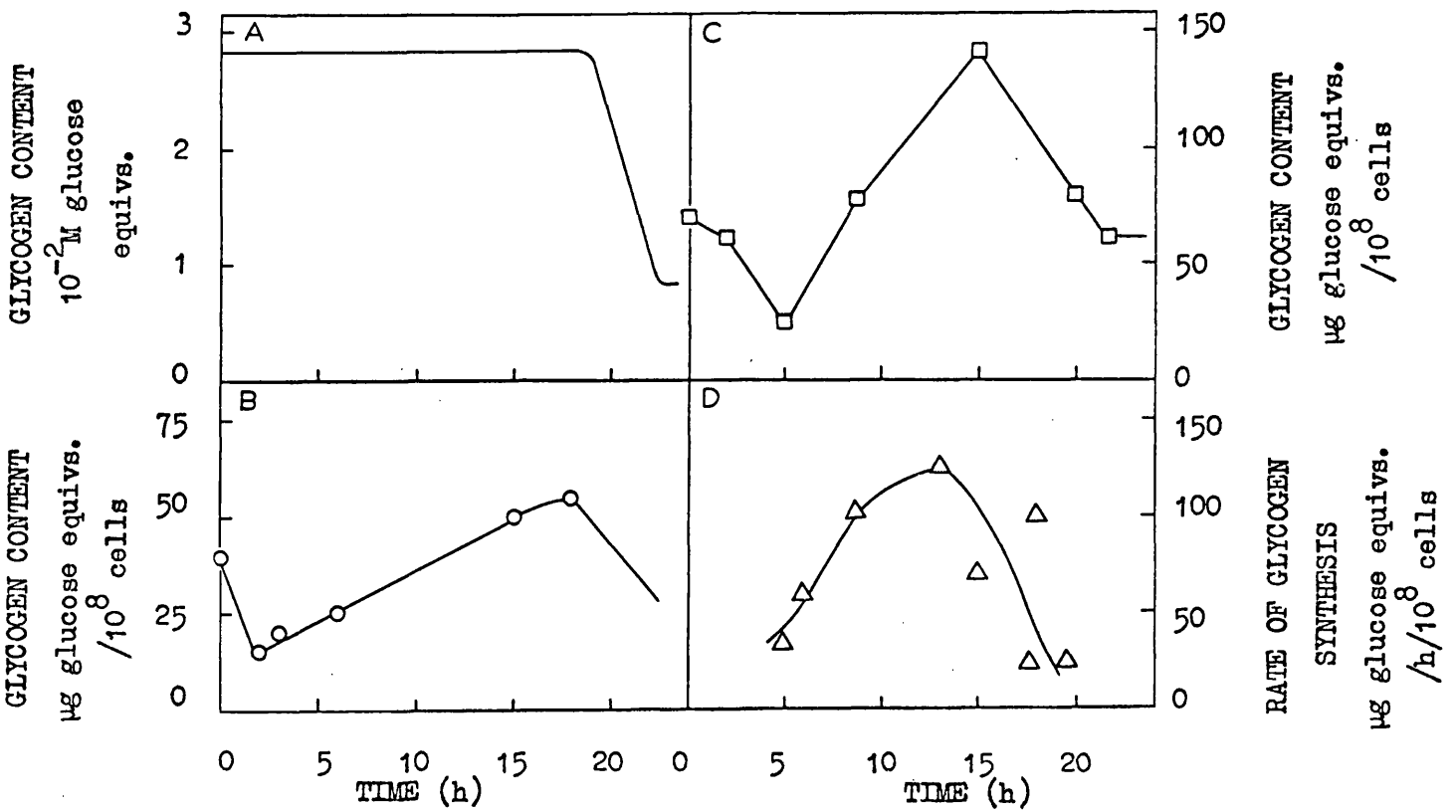


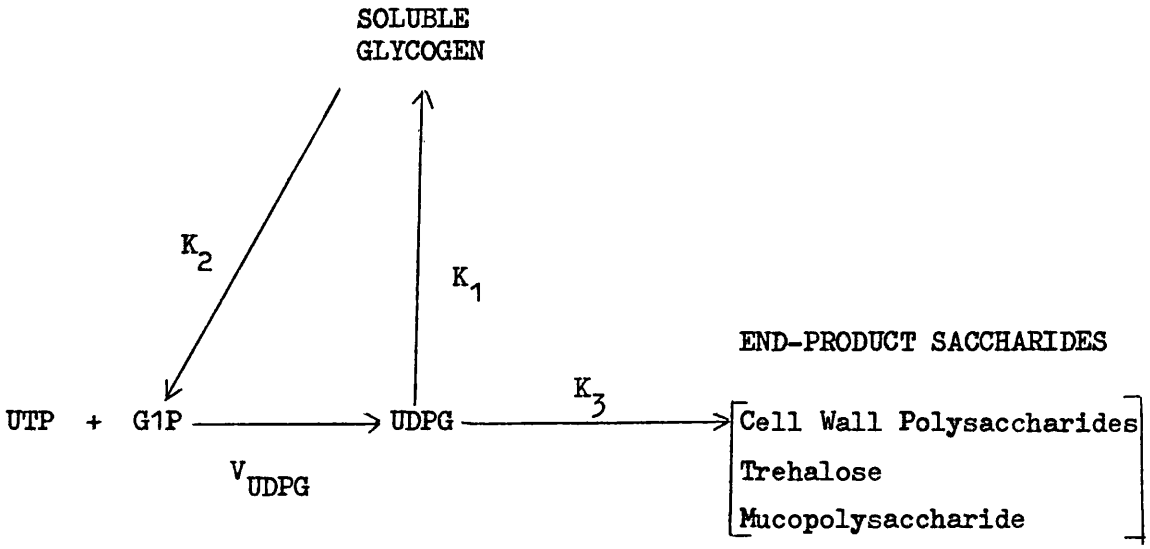
FIG. 15 Changes in Cellular Glycogen Content and The Rate of Glycogen Synthesis during The Development of D.discoideum Myxamoebae.

A : Changes in the cellular content of non cell wall glycogen during the development of bacterially-grown myxamoebae (from Wright et al. 1968)

B : Changes in the cellular content of non cell wall glycogen during the development of bacterially-grown myxamoebae (from Sussman @ Sussman 1969)

C : Changes in the cellular content of non cell wall glycogen during the development of axenically-grown myxamoebae initially containing 0·074mg glycogen/10^8 cells (Chapter 2)

D : Variation in the rate of glycogen synthesis during the development of axenically-grown myxamoebae initially containing 2·63mg glycogen/10^8 cells (from Hames, Weeks @ Ashworth 1972)



"where V_{UDPG} = the rate of UDPG synthesis (catalysed by UDPG pyrophosphorylase) determined according to the Michaelis Menten equation, and K_1 - K_3 are rate constants". (Wright et al., 1968b)

Seven more assumptions are implicit in the model:

"1. The K_m values (of UDPG pyrophosphorylase) for G1P and UTP ... determined in vitro apply in vivo and do not change from aggregation to sorocarp.

2. The rate of synthesis of UDPG follows Michaelis Menten kinetics. Binary complexes have been assumed.

3. The rates of synthesis of soluble glycogen and end-product saccharides are directly proportional to the concentration of UDPG. This is in accord with Michaelis Menten kinetics if K_{UDPG} (the Michaelis constant for UDPG) is much larger than the concentration of UDPG. This is known

to be the case in the synthesis of both soluble glycogen and cell wall glycogen.

4. The rate of production of G1P is directly proportional to the concentration of soluble glycogen.

5. All the reactions are essentially irreversible under the conditions existing within the cell.

6. The end-product saccharides are not degraded in the sorocarp.

7. There is no gluconeogenesis."

(Wright et al., 1968b)

Data obtained on in vivo levels of metabolites and reaction rates and in vitro kinetic parameters of enzymes were supplied to a PDP-8 computer programmed according to this model, and alterations were made in various reaction parameters until the changes in metabolite levels, reaction rates etc., actually observed in vivo during development were mimicked by the computer. The successful solution was that the observed in vivo changes would occur under the following conditions:

1. If a closed cycle exists prior to culmination in which glycogen is continually broken down (reaction K_2) and resynthesised (reaction K_1) as depicted above (p.35).

2. If the rate of glycogen degradation (reaction K_2) triples in a linear fashion from aggregation to culmination.

3. If at culmination the flux through K_1 ceases and is diverted instead through reaction K_3 to synthesise end-products.

Criticisms of the Model

Whilst the technique of computer analysis of metabolism is potentially very rewarding, the type of analysis outlined above is open to severe criticism.

Firstly, the solution eventually found is undoubtedly only one

of several possible; the fact that it fulfils all criteria presently known is no proof of its operation *in vivo*.

Secondly, the numerical data used in this analysis is of doubtful validity. Thus, on the basis of Wright's own experiments, levels of metabolites such as UDPG and G6P, and even rates of synthesis and degradation of glycogen, vary widely from experiment to experiment during the development of bacterially-grown myxamoebae - standard errors of 60% are not uncommon (Pannbacker, 1966). In any case, as pointed out by Newell & Sussman (1969), "one may also question the relevance of computations based on total intracellular concentrations of metabolites since the latter carries the implicit assumption that the compounds are distributed uniformly within the cell. In the slime moulds, polysaccharide synthesis and degradation are thought to be compartmentalised in vesicles (*see later*) and estimates of total cell (rather than local organelle) concentrations of these metabolites seem to be uncertain parameters".*

Thirdly, the model assumes that UDPG pyrophosphorylase, a key enzyme in the computer model, increases only 3-fold in specific activity during development, whereas there is now much evidence to suggest that the figure is nearer 8-10 fold (Newell & Sussman, 1969; Edmundson & Ashworth, 1972). Moreover, the former workers have presented evidence

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* Whilst this is a serious criticism of the model of Wright *et al.* (1968b), it is unfortunately true that, at present, we are not able to determine accurately local concentrations of molecules and so data and discussions in the rest of this thesis are forced to refer to total intracellular concentrations of molecules.

that UDPG pyrophosphorylase is polymorphic (possibly each species has different kinetic properties), whereas the model assumes that only one species is present during the development of D. discoideum.

Fourthly, the qualitative changes in the glycogen concentration of bacterially-grown myxamoebae during development are not clear: Whilst Wright and her coworkers claim that cellular glycogen is conserved throughout development until culmination when it decreases (Wright & Dahlberg, 1965; Fig. 15A), White and Sussman described the completely different picture of glycogen degradation followed by resynthesis and final degradation at culmination (see above; Fig. 15B ; White & Sussman, 1961). These differences in results are not completely attributable to experimental technique since although Wright & Dahlberg determined cellular glycogen in terms of a 100,000 g pellet and White & Sussman followed alkali soluble, ethanol insoluble hexose, the percentage of the 100,000 g glycogen pellet that is alkali soluble remains constant throughout development (Wright & Dahlberg, 1967).

Clearly, if White & Sussman are correct and myxamoebal glycogen does not remain at a constant level until culmination, then the detailed model of Wright is incorrect.

The results presented in this chapter, obtained using axenically-grown myxamoebae, support the findings of White & Sussman (1961, 1963a) using bacterially-grown myxamoebae, and prove conclusively that the detailed model of Wright et al. (1968b) is untenable at least as regards the development of axenically-grown myxamoebae.

Thus we have shown that the axenically-grown myxamoebae do not conserve but rapidly degrade their myxamoebal glycogen irrespective of the glycogen level and that axenically-grown myxamoebae containing low

levels of glycogen exhibit a pattern of glycogen metabolism (Fig. 15C) identical to that reported by White & Sussman for bacterially-grown myxamoebae (White & Sussman, 1961; Fig. 15B). Whereas White & Sussman (1961) attributed the initial glycogen degradation to the final utilisation of bacterial glycogen, it seems much more likely from our results that it is myxamoebal glycogen that is degraded.

Therefore, one critical datum used in the derivation of Wright's model - that myxamoebal glycogen is conserved until fruiting body formation - appears to be incorrect.

However, if glycogen is indeed synthesised during development, then considerable gluconeogenesis must occur since the amount of glycogen synthesised during the development of axenically-grown myxamoebae is far greater than that present in vegetative myxamoebae and this synthesis occurs at a time when the glucose pool is very low and constant (see RESULTS). Now, a basic assumption of Wright's model is that no gluconeogenesis occurs (assumption no. 7); yet it is significant that even Wright and her coworkers are not clear about the extent of gluconeogenesis.

In 1960(a) Wright and Anderson stated that extensive carbohydrate synthesis from the carbon skeleton of amino acids occurs in the later stages of development; in 1964 Wright et al. even argued that gluconeogenesis begins after 10-12 h of development; and as late as 1967, Pannbacker and Wright stated "we find that ^{14}C-labelled glutamic acid is not only oxidised but is incorporated into cellulose at least 25% as well as ^{14}C-labelled glucose. These facts indicate that we are dealing with a system of gluconeogenesis in which protein is an

important substrate". However, upon presentation of the computer model (Wright et al., 1968b), this view dramatically changed to "the quantitative importance of gluconeogenesis in this system, although minor, remains to be determined.", and by 1970, Jones & Wright were stating that "gluconeogenesis does not occur to any significant extent during development".

Let us examine the evidence for and against gluconeogenesis: The evidence quoted as being against gluconeogenesis occuring during development (Wright et al., 1968b) comes from three sources, (1) that the anthrone reactive material content of developing cells, grown bacterially, remains constant at 400-500 μg glucose equivalents/10^8 cells (White & Sussman, 1961), (2) that incorporation of ^{14}C-amino acids into end-product saccharides is negligible (Cleland & Coe, 1969), and (3), that fructose 1,6-diphosphatase (FDPase) appears to be the rate limiting enzyme for gluconeogenesis and in vivo may not be sufficient to catalyse significant gluconeogenesis (Baumann & Wright, 1969; Cleland & Coe, 1968). Let us consider these in turn.

(1) There are two main points regarding the "constancy" of the cellular content of anthrone reactive material during the development of bacterially-grown myxamoebae (White & Sussman, 1961). Firstly, unlike axenically-grown myxamoebae, during the development of bacterially-grown myxamoebae, the amount of glycogen synthesised approximately equals the vegetative myxamoebal glycogen content and so perhaps one should not expect a net increase in anthrone-positive material during this period. However, a second point is that vegetative myxamoebal glycogen is degraded and only later resynthesised so that one should observe a fall in total anthrone-positive material followed by an increase back to

vegetative myxamoebal levels. Unfortunately, White & Sussman (1961) made no measurements of total anthrone-positive material between 0 h and 20 h developmental time and therefore no conclusions can be drawn from these data about the presence or absence of gluconeogenesis during this period. Indeed, Gregg & Bronsweig (1956) and Ceccarini & Filosa (1965) observed a significant increase in the total reducing substances/ dry weight during the development of bacterially-grown myxamoebae and concluded that significant gluconeogenesis does occur during this phase.

(2) The data of Cleland & Coe (1969) were obtained by harvesting bacterially-grown cells at the preculmination stage, exposing them to (^{14}C)aspartate, and then allowing them to fruit. The results certainly appear to indicate that gluconeogenesis is only slight from preculmination onwards but give no information on the extent of gluconeogenesis prior to preculmination, the period during which glycogen synthesis, and presumably gluconeogenesis is occurring. This data also conflicts with that of Pannbacker & Wright (1967) who showed that the carbon of (^{14}C)glutamate is incorporated into cellulose at least 25% as well as carbon from (^{14}C) glucose.

(3) Finally, measurements of the activity of FDPase (Cleland & Coe, 1968) do not at all discount the presence of active gluconeogenesis during development. Cleland & Coe (1968) working with bacterially-grown myxamoebae state that there is sufficient FDPase to yield

" a potential rate of glucose production through this step of about 1 mg/h per 100 mg protein. Although precise estimates of the rate of glucose incorporation into stable polysaccharides are not available,

a rise in alkali-insoluble carbohydrate* ... during the 10 h culmination would require a rate of glucose synthesis of about 0.41 mg/h per 100 mg protein. Since the enzyme rate is given in terms of soluble protein while the latter rate is calculated from total protein, fructose diphosphatase may be the rate limiting step in the gluconeogenic sequence". Obviously, in the absence of figures for the proportion of total protein that is soluble, it is impossible to accurately evaluate the potential activity of FDPase during the development of bacterially-grown myxamoebae but it is clear that sufficient activity may exist for active gluconeogenesis. This is probably also true for the development of axenically-grown cells since the level of FDPase in these cells ** is at all times equal to or greater than that during the development of bacterially-grown cells (Fig. 16).

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* Equivalent to cell wall polysaccharide

** It is interesting that myxamoebae grown in axenic medium containing added glucose contain much less FDPase than cells grown in axenic medium lacking added glucose (Fig. 16). That this difference is not due to the presence of activators or inhibitors is indicated by the fact that the activities of mixtures of cell extract were found to be the sums of the activities displayed by the separate extracts (Table 10). Although this does not rule out the presence of enzyme bound inhibitors or activators not in excess, it suggests that the difference in activities is probably due to differences in cellular enzyme content, that is, exogenously supplied glucose can by some unknown mechanism reduce the cellular content of FDPase.

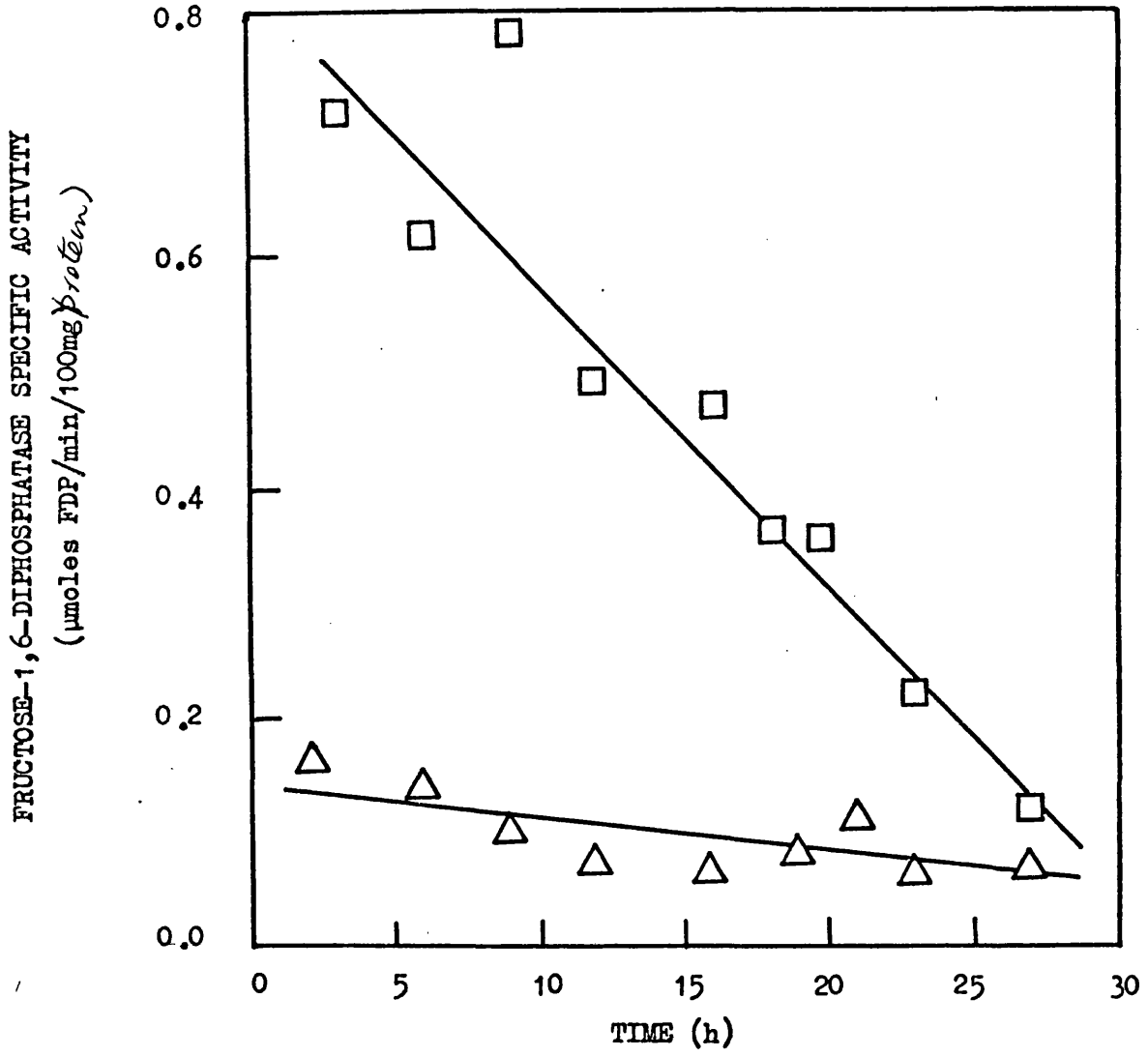


FIG. 16 Variation in Fructose-1,6-Diphosphatase Activity during The Development of Axenically-grown Myxamoebae.

□; myxamoebae initially contained 0·30mg glycogen/10^8 cells

△; myxamoebae initially contained 5·09mg glycogen/10^8 cells

TABLE 10 To show the absence of excess amounts of activators or inhibitors of fructose 1,6-diphosphatase in cell extracts of axenically-grown myxamoebae during the developmental phase

*Sample	Measured fructose 1,6-diphosphatase activity (ΔOD_{340}/min)	Expected fructose 1,6-diphosphatase activity (ΔOD_{340}/min)
NS 3	0.029	-
G 3	0.008	-
NS 3 + G 3	0.036	0.037
NS 15	0.022	-
G 15	0.008	-
NS 15 + G 15	0.026	0.030

* Each sample is denoted in terms of initial myxamoebal glycogen content (NS or G - see below) and the developmental time of sampling (in h).

NS: myxamoebae initially containing 0.302 mg glycogen/10^8 cells

G : myxamoebae initially containing 5.090mg glycogen/10^8 cells

Not only this, but there is evidence that the true *in vivo* activity of FDPase may not be assayed for by the *in vitro* assays used. Purified *D. discoideum* FDPase has a peak activity at pH 9.5 but another equal peak of activity occurs at pH 7.5 in the presence of 0.2 mM EDTA (Baumann & Wright, 1969), although EDTA has no effect on the activity of the enzyme at pH 7.5 in crude extracts, (Cleland & Coe, 1968; Baumann & Wright, 1969). Traniello et al.,(1971, 1972) have recently found that liver FDPase exists in two distinct forms, one with a pH optimum at pH 9.5 ("alkaline" enzyme) and another with a pH optimum at pH 7.5 ("neutral" enzyme) and "neutral" enzyme is converted to the "alkaline" enzyme by proteolytic digestion. It is therefore possible that FDPase assayed in crude extracts is not the *in vivo* active enzyme but an altered (degraded?) form of it.

Clearly, there is no conclusive evidence for the absence of gluconeogenesis at *all* stages of development - on the contrary, the results presented in this chapter strongly suggest that significant gluconeogenesis does occur prior to culmination.

So far, I have shown how studies of the development of both axenically and bacterially-grown myxamoebae fail to support all the assumptions of the model of Wright *et al*. (1968). Other data, presented in this chapter, are in disagreement with the predictions of the model.

Thus, the second condition for the operation of the Wright model *in vivo* (p. 36 above) (Wright *et al*., 1968b) is that the rate of degradation of glycogen triples in a linear fashion from aggregation to culmination. Since prior to culmination, a closed cycle exists such

that the rate of glycogen breakdown is balanced by its rate of synthesis (Wright et al., 1968b), this predicts that the rate of glycogen synthesis should also triple in a linear fashion from aggregation to culmination. Indeed, Marshall et al. (1970) have measured the rate of synthesis and found that its value at culmination is threefold that at aggregation, although no study of the kinetics of its increase have been made, and that the rate of glycogen synthesis approximately equals the rate of glycogen turnover at these two stages. In contradiction to these findings, during the development of axenically-grown myxamoebae containing low levels of myxamoebal glycogen, there is a net synthesis of glycogen prior to culmination and therefore the rate of glycogen synthesis must exceed the rate of glycogen degradation, i.e. a closed cycle of glycogen synthesis and degradation cannot possibly exist at this time. Moreover, measurements of the rate of glycogen synthesis during the development of axenically-grown myxamoebae initially containing high levels of myxamoebal glycogen indicate that the rate of glycogen synthesis does not triple in a linear fashion from aggregation to culmination but rather peaks at aggregation and declines before culmination (Figure 15D).

The data discussed above indicate that during the development of axenically-grown myxamoebae, glycogen metabolism follows the pattern described by White & Sussman for the development of bacterially-grown myxamoebae, that myxamoebal glycogen is not conserved, that gluconeogenesis probably occurs, that a closed cycle of glycogen metabolism does not exist, and that the rate of glycogen synthesis peaks at aggregation rather than tripling in a linear manner from aggregation to culmination. All this data is inconsistent with the detailed model of Wright described above.

B Two Pools of Glycogen?

Evidence is presented in this chapter that supports the hypothesis that axenically-grown myxamoebae and, by analogy, bacterially-grown myxamoebae, possess two separate pools of glycogen during the developmental phase of the life cycle.

Thus vegetative myxamoebal glycogen is extremely variable in level according to growth conditions, is present in the cytoplasm as non-membrane-bounded granules, and is rapidly degraded during development to products which are oxidised to carbon dioxide. This degradation is not caused by phosphorylase action, since phosphorylase activity is far too low at this time, but is probably due to amylase activity. Degradation takes place at a linear rate irrespective of initial glycogen concentration. (This is possibly caused by the linear decrease in amylase activity which occurs during the same period (this chapter; Fig.12), maintaining a constant glycogen-amylase ratio).

However, although axenically-grown myxamoebae contain active amylases irrespective of vegetative myxamoebal glycogen content, myxamoebae containing less than 0.1 mg glycogen/10^8 cells synthesise glycogen during development ("developmental glycogen") and evidence has been presented from isotopic labelling experiments that this glycogen is synthesised even when the developing cells contain large amounts of myxamoebal glycogen undergoing degradation. Developmental glycogen may thus be unavailable to amylase activity and indeed another glycogen degradative enzyme, phosphorylase, is synthesised and peaks in activity at the time of developmental glycogen degradation.

The degradation of myxamoebal glycogen does not appear to be a developmentally important event since myxamoebae develop normally

irrespective of its level or its degradation rate. The amylases responsible for its degradation may thus merely be "scavenger" enzymes whose role is to remove unwanted cytoplasmic carbohydrate reserves prior to fruiting body formation. However, developmental glycogen is synthesised at a specific phase of development, synthesis occurs irrespective of the total cellular content of glycogen, and degradation only occurs at culmination and may require the synthesis of a specific phosphorylase. Synthesis and degradation of developmental glycogen may thus be developmentally important events.

The question inevitably arises as to how the developing cell can separate two pools of glycogen. One way that this could occur is if two different species of glycogen molecule were synthesised. Many systems are known where at least two fractions of glycogen, different structurally and/or in their susceptibility to specific enzymic digestion are present in the same cell (Trevelyan & Harrison, 1956; Holme et al., 1958; Eaton, 1961). Indeed, as Pannbacker & Wright (1967) observed, the reserve carbohydrates of many protists exist in a variety of metabolically distinct pools. In the case of slime mould development, it would be sufficient to separate two pools of glycogen if myxamoebal glycogen was susceptible to amylase attack and developmental glycogen was insensitive to amylase but degradable by phosphorylase.

There is some evidence in agreement with this hypothesis. Thus, we have shown in this chapter that vegetative myxamoebal glycogen is readly degraded by cellular amylase activity. However, White & Sussman (1963a) showed that glycogen synthesised during the development of bacterially-grown myxamoebae (developmental glycogen) is insensitive

to β-amylase activity (provided that the glycogen is prepared by a mild procedure not involving hot alkali treatment), although it is degraded by α-amylase. To my knowledge, no detailed study has been made of the products of D. discoideum amylase action and thus it is not possible to state unequivocally the type of amylase present. However, the data presented in this chapter indicate that the amylase present during the developmental phase of axenically-grown myxamoebae is approximately 2.5 times more active at pH 4.5 than at pH 6.9, and this is characteristic of β-amylase (Bernfeld, 1955). This does not rule out the presence of α-amylase; indeed, Rosness (1967) and the data of Jones & Wright (1970) suggest that α-amylase activity increases just prior to fruiting body construction (the time at which developmental glycogen is degraded).

Alternatively, spatial separation of glycogen pools could occur; that is, myxamoebal glycogen may be present in a different cellular compartment from developmental glycogen. Electron microscopical evidence presented in Chapter I indicates that myxamoebal glycogen is cytoplasmic and unbounded by membranes. The question is, therefore, whether developmental glycogen is enclosed in some membranous compartment isolated from amylase action. Cells of D. discoideum are known to possess an extremely complex membranous system at all stages of development (e.g. Hohl & Hamamoto, 1969; Gregg & Badman, 1971; George et al., 1972) but no particular compartment has yet been identified to specifically contain glycogen, although there is evidence that certain unidentified carbohydrates are extremely localised during the phase of development when developmental

glycogen is present (Bonner et al., 1955).

Even though two pools of glycogen may exist, they are not completely separate since, given that developmental glycogen is used for end-product saccharide synthesis (an unproven fact in my opinion), some myxamoebal glycogen can also be used for this purpose when the vegetative myxamoebal glycogen pool is large enough.

C The Control of Developmental Glycogen Synthesis

During the development of myxamoebae initially containing high levels of myxamoebal glycogen, the rate of glycogen synthesis increases to a peak at aggregation and then decreases. However, during this time, glycogen synthetase is decreasing in activity, and, further, there is at all times an excess of enzymic activity (Weeks, in Hames, Weeks & Ashworth, 1972). Thus the alteration in the rate of glycogen synthesis is apparently not caused by alteration in total cellular glycogen synthase content.

However, Rosness et al. (1971) have shown that the glycogen synthetase of D. discoideum probably exists in two forms as has been described for other systems (Rothman-Denes et al., 1970; Tillez-Inon & Torres, 1970; Whelan, 1967). Total glycogen synthetase activity decreases during development but there is a quantitative conversion of G6P independent enzyme to G6P dependent enzyme. This has been interpreted as a mechanism to facilitate the switching off of glycogen synthesis as G6P falls to undetectable levels during fruiting body construction (Rosness et al., 1971) but it should be noted that the

conversion begins at aggregation so that, in the absence of a complete knowledge of the properties of the two forms of glycogen synthetase, one cannot rule out the possibility that another reason for the conversion is to facilitate the synthesis of developmental glycogen once aggregation is complete.

The rate of glycogen synthesis at any given cellular glycogen synthetase content will depend on the cellular concentrations of the substrates UDPG and G6P. Wright (1966), Wright & Dahlberg (1967), and Marshall et al. (1970) have argued that the increase in the rate of glycogen synthesis prior to culmination is caused by increases in the size of these substrate pools. In agreement with this, in the next chapter, it will be shown that the cellular pool of UDPG varies in a manner qualitatively similar to the variation in the rate of glycogen synthesis. Also, in vivo levels of UDPG (8×10^{-4} M; Chapter III) are below the K_m of glycogen synthetase for UDPG (4.64×10^{-3} M; derived from the data of Weeks & Ashworth, 1972). In contrast, the G6P level remains fairly constant during developmental glycogen synthesis (Chapter III). It is therefore possible that the rate of glycogen synthesis during development, as during growth (Weeks & Ashworth, 1972), is controlled by metabolite levels (in particular, by the cellular concentration of UDPG) rather than by changes in the cellular enzyme content, in agreement with the contention that "changes in the levels of certain essential enzymes are not significant with respect to controlling the metabolite flux necessary for the accumulation of specialised end-products" (Wright, 1970). Control of glycogen synthesis by the size of the intracellular UDPG pool has also been

inferred for glycogen accumulation by other organisms, e.g. Agrobacter (Pannbacker & Wright, 1967).

The developmental phase of D. discoideum may therefore be a case where the control of developmental glycogen synthesis occurs mainly at the substrate, certainly the metabolic, level whereas the regulation of developmental glycogen degradation may be located primarily at the protein synthetic level (phosphorylase synthesis). However, it is clear that even protein synthetic controls are subject to modulation by metabolite levels since the peak specific activity of phosphorylase is a function of the glycogen content of the developing cells. Similar examples of metabolite levels inducing the synthesis of eukaryotic cell enzymes are known (e.g. Schimke , 1969) but it is especially interesting that in the case of D. discoideum development, induction of developmental enzymes appears to be an "all or none" phenomenon.

Thus, Newell et al. (1972) have shown that, if developing cells which have accumulated certain developmental enzymes are dissociated and then allowed to reassociate, a complete second round of synthesis of these enzymes occurs! Further dissociation and reassociation results in a complete third round of enzyme synthesis. Synthesis of these enzymes during development therefore occurs in quanta and the phenomenon has been called quantal control (Sussman & Newell, 1972). Quantal control has been demonstrated not only by cell dissociation experiments but also during the inhibition of slug migration by exposure to overhead light (Sussman & Newell, 1972) and during the growth phase of axenically-grown myxamoebae (Ashworth & Quance, 1972).

Phosphorylase is one of the enzymes which is known to be subject to quantal control on the basis of cell dissociation experiments

(Firtel & Bonner, 1972). The accumulation of twice the peak specific activity of phosphorylase in developing cells initially containing 6.87 ± 0.91 mg glycogen/10^8 cells as in cells containing 0.32 ± 0.06 mg glycogen/10^8 cells may therefore be yet another demonstration of the phenomenon, although the necessary experiments to show that at intermediate levels of glycogen intermediate levels of phosphorylase are not accumulated have not been done.

SUMMARY

1. The metabolism of glycogen has been investigated during the development of myxamoebae initially containing levels of glycogen between 0.046 and 5 mg/10^8 cells.

2. Evidence has been obtained that developing cells of D. discoideum contain two incompletely separate pools of glycogen; a pool of glycogen is present in vegetative myxamoebae ("myxamoebal glycogen") and is rapidly degraded during development by cellular amylase activity to products which are oxidised to carbon dioxide, whilst another pool of glycogen is synthesised during development ("developmental glycogen") probably using hexose derived via gluconeogenesis and only degraded during fruiting body formation, possibly by phosphorylase which is synthesised at this time.

3. The data described above, and relevant results of other workers, has been discussed in relation to the computer model of differentiation devised by Wright et al. (1968b).

CHAPTER III

THE CONTROL OF END-PRODUCT SACCHARIDE SYNTHESIS DURING DEVELOPMENT

CHAPTER III

THE CONTROL OF END-PRODUCT SACCHARIDE SYNTHESIS DURING DEVELOPMENT

INTRODUCTION

As previously described, development in the cellular slime mould D. discoideum is accompanied and characterised by the synthesis of certain carbohydrates [notably trehalose, mucopolysaccharide, and cell wall polysaccharide (p.iv)] not present to any large extent in the vegetative phase of the life cycle (White & Sussman, 1961; 1963b; Ceccarini & Filosa, 1965). The enzymes involved in the synthesis of these end-product saccharides (p.iv) and the mechanisms whereby their activity is controlled have been intensely studied in the hope that such investigations will reveal the control mechanisms which regulate cellular differentiation in this and, by analogy, other, organisms.

Thus, Sussman & Sussman (1969) have reviewed the evidence for the existence of transcriptional and translational controls of the de novo synthesis of some enzymes involved in the synthesis of these end-product saccharides. The increased rates of synthesis of these enzymes during development is presumably a means whereby the metabolic flux through the synthetic pathway may be increased, leading to the differentiated state. Wright (1966) has pointed out, however, that an alteration in the cellular concentration of an enzyme will only cause an alteration in overall metabolic flux if the enzyme concerned is catalysing the rate limiting step of the overall metabolic sequence

and is working at its maximal velocity. If the second of these two conditions is not satisfied it would be possible to increase the overall metabolic flux by alterations in the concentration of the substrate and/or effectors of that enzyme.

Determination of the *in vivo* controlling factors of any one pathway is complicated by the fact that it is experimentally difficult to alter the flux through that pathway.

In the previous chapter, it has been shown that the majority of axenic myxamoebal glycogen is rapidly degraded during development and oxidised to carbon dioxide. However, measurements of total cellular carbohydrate indicate that some myxamoebal glycogen may be used for end-product saccharide synthesis and that the amount of end-product saccharides synthesised may be a function of the myxamoebal glycogen content (Chapter II, p. 28) and therefore a study (reported in this chapter) was undertaken:

(1) to investigate in detail the relationship between myxamoebal glycogen content and the synthesis of end-product saccharides;

(2) if the fluxes through end-product pathways were indeed a function of myxamoebal glycogen content, to use this phenomenon to probe the controls of end-product saccharide synthesis.

MATERIALS

Galactose, UDPG, G6P, phosphoenolpyruvate, pyruvate kinase, peroxidase, and O-dianisidine were purchased from Sigma (London) Ltd., London W.5, U.K. All other chemicals were of the highest purity commercially available and obtained from the sources indicated in Chapters I and II, or from BDH Chemicals Ltd., Poole, Dorset, U.K., or Fisons Scientific Apparatus Ltd., Lougborough, Leics. U.K.

METHODS

Determination of Total Cellular Carbohydrate Content

Total cellular carbohydrate content was determined as described in Chapter II.

Determination of Cellular Glycogen Content

Cellular glycogen content was determined as described in Chapter II.

Determination of Mucopolysaccharide

Cell extracts were prepared as described above, 9 ml of extract being required for each mucopolysaccharide determination.

5.2 ml of 0.3 M $Ba(OH)_2$ was added to 4.5 ml of cell extract and then 4.0 ml of 5% (w/v) $ZnSO_4$ was added, mixing the solution well after each addition. Insoluble material was removed by centrifugation at 1000 g for 15 min. The supernatant was evaporated to dryness at 50°C under vacuum and the residue dissolved in 2 ml water. After neutralisation, using KOH, a sample of this solution was assayed for free galactose and galactosamine (pre-hydrolysis sample) using the galactose oxidase assay (Roth et al., 1965), except that 0.3 ml of 1% O-dianisidine in 95% ethanol was used in the assay mixture instead of benzidine, and optical density readings were made at 400 nm instead of 295 nm twenty minutes after the acidification which is carried out to stop the reaction. This procedure was found to give more reproducible results than that described by Roth et al. (1965) and gave no cross reaction with up to 30 mM glucose.

0.5 ml of 10N hydrochloric acid was added to another 4.5 ml of the same cell extract and the mucopolysaccharide hydrolysed at 95°C for 4 h. After cooling in ice for 10 min, insoluble material was removed by centrifugation at 1000 g for 15 min, the supernatant was evaporated at 50°C under vacuum and the residue was dissolved in 3 ml of water. After neutralisation, 2.6 ml of 0.3 M $Ba(OH)_2$ and then 2.0 ml of 5% (w/v) $ZnSO_4$ were added to the solution, mixing well after each addition. The solution was left in ice for 30 min and then centrifuged and evaporated to dryness as before. The residue was then dissolved in 2 ml of water and a sample assayed for free galactose and galactosamine (post-hydrolysis sample) using the galactose oxidase method described above.

The difference in free galactose and galactosamine content of the pre- and post-hydrolysis samples represented the amount of mucopolysaccharide present in the sample.

Determination of Cell Wall Polysaccharide

1 ml 90% (w/v) KOH was added to 2 ml of cell extract and the suspension heated at 100°C for 20 min. Insoluble material was removed by centrifugation at 17,000 g for 10 min and extracted with 1 ml 30% (w/v) KOH at 100°C for a further 20 min. After recentrifugation, the pellet was washed twice with 2 ml of water each time, and finally dissolved in 67% (v/v) H_2SO_4 (2 h at room temperature). A sample of this solution was then assayed for hexose content by the anthrone method of Hassid & Abraham (1957).

Determination of Trehalose

Trehalase was prepared from Neurospora crassa by a modification of the method of Hill & Sussman (1963) as described in Appendix A. The enzyme was specific for trehalose when tested on a large range of carbohydrates including D. discoideum glycogen, galactose, maltose, cellobiose, and cellulose (see Appendix A).

The procedure for determination of the trehalose content of a cell sample was as follows. Cells were harvested at various stages of development into ice cold water, ultrasonicated as described above, and then 0.25 ml of ice cold 60% perchloric acid immediately added to 4.5 ml of the cell extract. The mixture was incubated at 0°C for 30 min and the protein precipitate then removed by centrifugation (1000 g, 20 min). The supernatant was neutralised using KOH, the precipitate of potassium perchlorate removed by centrifugation (1000 g, 10 min), and the supernatant assayed for trehalose. Each assay mixture contained 0.8 ml of neutralised perchloric acid extract, 0.1 ml 0.5 M phosphate buffer, pH 5.6, and 0.1 ml of purified trehalase. This was incubated for 4 h at 37°C, boiled for 10 min to stop the reaction, and the glucose content of the solution estimated by the hexokinase method of England & Randle (1967). This value was corrected for the glucose content of pre-trehalase extract, also determined using the hexokinase assay of England & Randle (1967).

Chromatography of Trehalose

0.25 ml saturated sodium sulphate and 10 ml absolute alcohol were added to 5 ml of neutralised perchloric acid extract, prepared as described above, and the mixture incubated at 0°C for

15 min. The precipitated glycogen was removed by centrifugation (38,000 g, 15 min) and the supernatant deionised by passage through Monobed MB3 resin. The resin was washed with an equal volume of water, the pooled solutions carefully evaporated to dryness under vacuum at 50°C, and the residue resuspended in 5 ml of water. This solution was deionised by passage through Monobed MB3 resin which was washed as before. The pooled solutions were evaporated to dryness at 50°C under vacuum using a Rotary Evapomix (Buchler Instruments Ltd., New York), resuspended in 0.1 ml and spotted onto Whatman No. 4 chromatography paper. The chromatogram was developed using isopropanol : n butanol : water (140 : 20 : 40) and carbohydrates were detected using alkaline silver nitrate (Trevelyan et al., 1950), and sodium metaperiodate(Evans & Dethier, 1957) and identified by comparison with authentic samples run on the same paper. Trehalose was eluted according to the method of Dimler et al. (1952) and concentrated at 50°C using a Rotary Evapomix, redissolved in 2 ml of water, and assayed as described previously.

Assay of Trehalase

Trehalase was assayed according to the method of Ceccarini (1966) using cell samples harvested in 0.016 M phosphate buffer, pH 6.0, and frozen at -70°C.

Assay of Trehalose 6-phosphate Synthase

The cells from 4 Millipores were harvested in 3 ml ice cold 10 mM tris, pH 7.5, containing 1 mM dithiothreitol, sonicated, and assayed immediately for trehalose 6-phosphate synthase activity by

measuring the formation of UDP as described by Roth & Sussman (1968).

Determination of Glucose 6-phosphate and UDP-glucose

Samples were prepared by harvesting the cells from 12 millipores in 4.5 ml ice cold water, adding 0.25 ml of ice cold 60% perchloric acid, and then sonicating with a 100 W MSE ultransonic disintegrator (peak to peak amplitude of 7 μm) for six 15 s periods with continuous cooling in an ice salt bath to prevent the temperature rising above 4°C. The entire procedure took approximately 10 min per sample.

Following sonication, the mixture was incubated for 30 min at 0°C and the resulting precipitate was removed by centrifugation. The supernatant was neutralised using ice cold 5N KOH, recentrifuged to remove insoluble potassium perchlorate, and then freeze dried. The residue was redissolved in 2 ml water and 0.2 ml aliquots used to assay G6P using the fluorimetric procedure described by Weeks & Ashworth (1972). The remainder of the sample was used for UDPG isolation and estimation as described by Weeks & Ashworth (1972) except that 10 ml of 1.5 M ammonium formate were used to elute UDPG from the Dowex 1 formate columns, and that each fluorimetric assay for UDPG contained 1 mmol of Tris chloride buffer, pH 8.5.

Determination of Sorocarp Glucose and Trehalose Distribution

Axenically-grown myxamoebae were allowed to develop as described previously. Mature fruiting bodies (40 h) were harvested rapidly in water and the suspension passed through 4 layers of wet

muslin, thus trapping the stalks but not the spores. The entrapped stalks were washed well and then resuspended in water. Stalk and spore suspensions were then analysed for glucose and trehalose as described previously.

RESULTS

End-Product Saccharide levels of Myxamoebae initially containing different Glycogen contents

1. Mucopolysaccharide

The mucopolysaccharide content of mature fruiting bodies is markedly influenced by the initial glycogen content of the myxamoebae; myxamoebae initially containing 5.590 ± 0.048 mg glycogen/10^8 cells synthesise twice the amount of mucopolysaccharide as myxamoebae initially containing 0.070 ± 0.008 mg glycogen/10^8 cells (Fig. 17, Table 11). No mucopolysaccharide is detectable until culmination. Synthesis of this polymer then occurs at similar rates irrespective of the myxamoebal initial glycogen content.

2. Cell Wall Polysaccharide

Axenically-grown myxamoebae strain Ax-2 contain a significant level of alkali-soluble carbohydrate which is maintained until fruiting body construction when cell wall polysaccharide synthesis occurs (Fig. 18). It is not known whether this alkali-insoluble carbohydrate present in the cells prior to culmination is chemically identical with cell wall polysaccharide.

During fruiting body construction, axenically-grown myxamoebae synthesise approximately the same amount of cell wall polysaccharide irrespective of their initial glycogen contents (Fig. 18, Table 11); thus 70-fold changes in myxamoebal glycogen content result in less than a 30% difference in the amount of cell wall polysaccharide accumulated.

3. Trehalose

Whereas during the development of bacterially-grown cells, trehalose remains at a low level until fruiting body construction (Ceccarini & Filosa, 1966), axenically-grown myxamoebae synthesise trehalose both during late aggregation and culmination (Fig. 19).

The trehalase of N. crassa is highly specific for trehalose as substrate (see Appendix A) but to confirm that the material assayed as trehalose in Fig. 19 is indeed this disaccharide, samples were chromatographed with authentic trehalose, eluted, and reassayed with trehalase. This procedure gave complete recovery of the presumed trehalose thus confirming its chemical nature.

The data in Fig. 19 and Table 11 show that the amount of trehalose synthesised by developing myxamoebae is extremely dependent on the initial glycogen content of the cells; four-fold variations in the mature sorocarp trehalose content can be obtained. In Fig.19 it can be seen that the accumulation of trehalose follows a biphasic pattern irrespective of the amount of trehalose accumulated. Whilst the final amount of trehalose accumulated was constant for a given myxamoebal glycogen content (Table 11), the 15-20 h plateau level of trehalose is subject to some variation.

4. Glucose

Most of the myxamoebal glycogen which is degraded during the development of axenically-grown myxamoebae has been broken down by 20 h but at this time a sudden rise is observed in the intracellular concentration of glucose (Fig. 20). Marked differences are observed

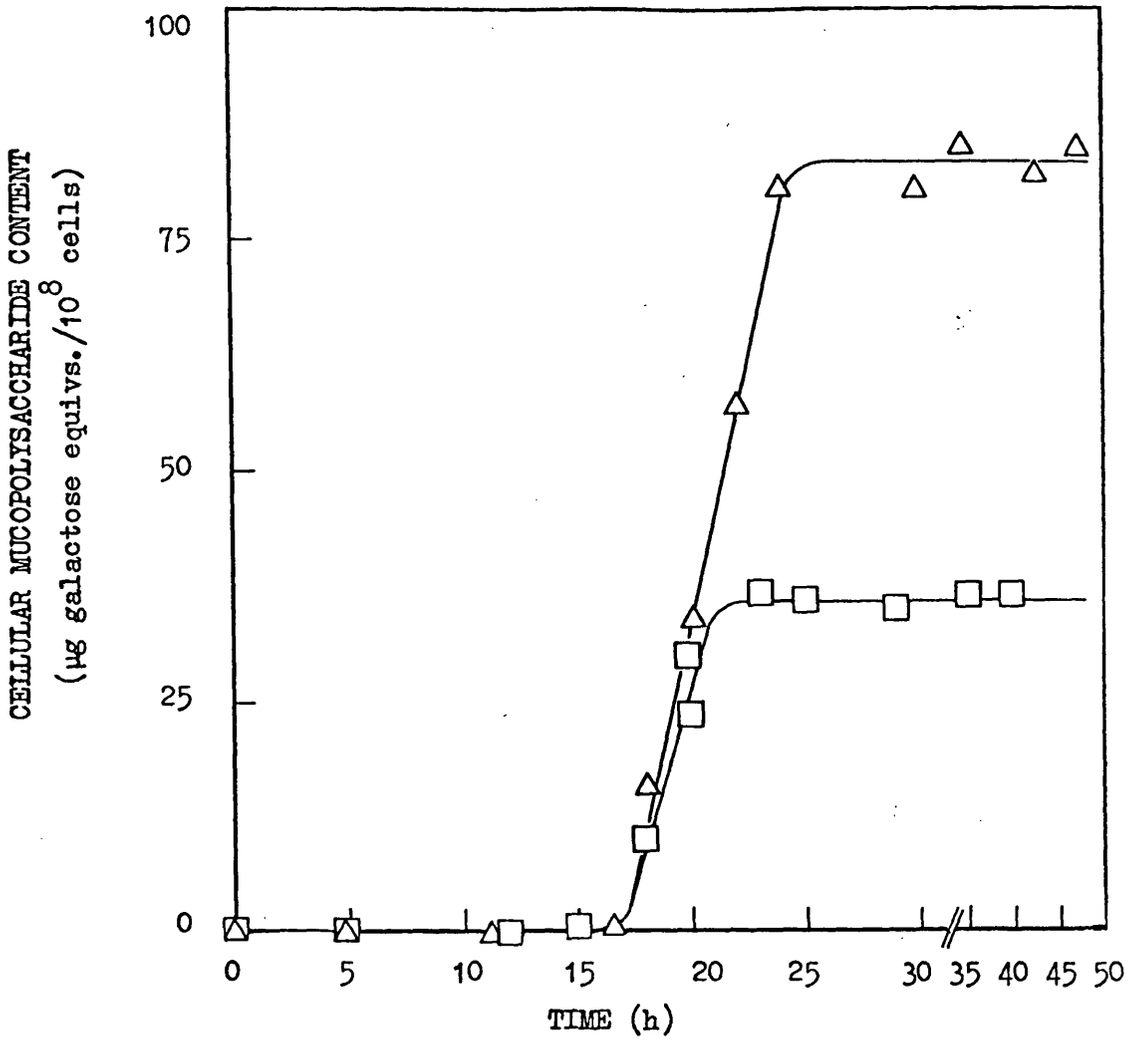


FIG.17 Accumulation of Acid Mucopolysaccharide during The Development of axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.06mg glycogen/10^8 cells

△ ; myxamoebae initially contained 5.65mg glycogen/10^8 cells

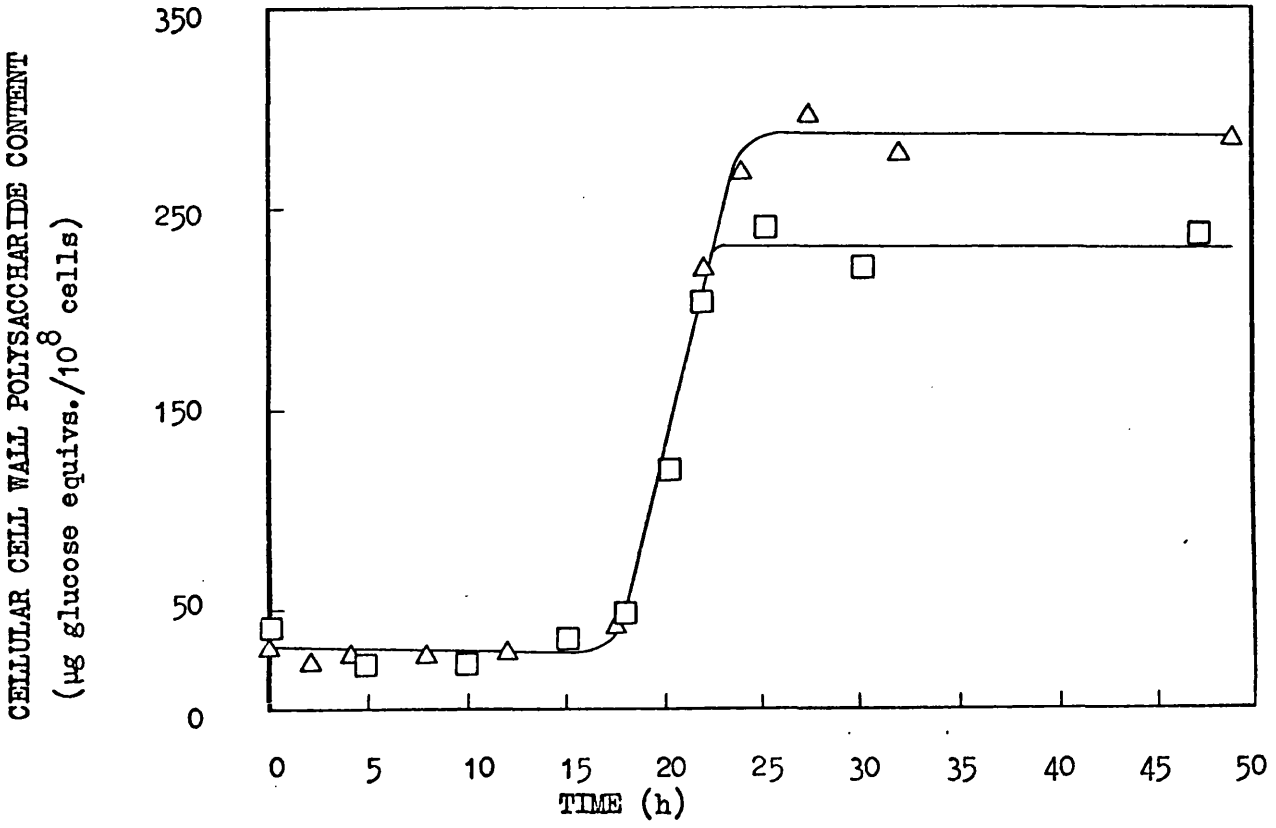


FIG. 18 Accumulation of Cell Wall Polysaccharide during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.07mg glycogen/10^8 cells

△ ; myxamoebae initially contained 5.70mg glycogen/10^8 cells

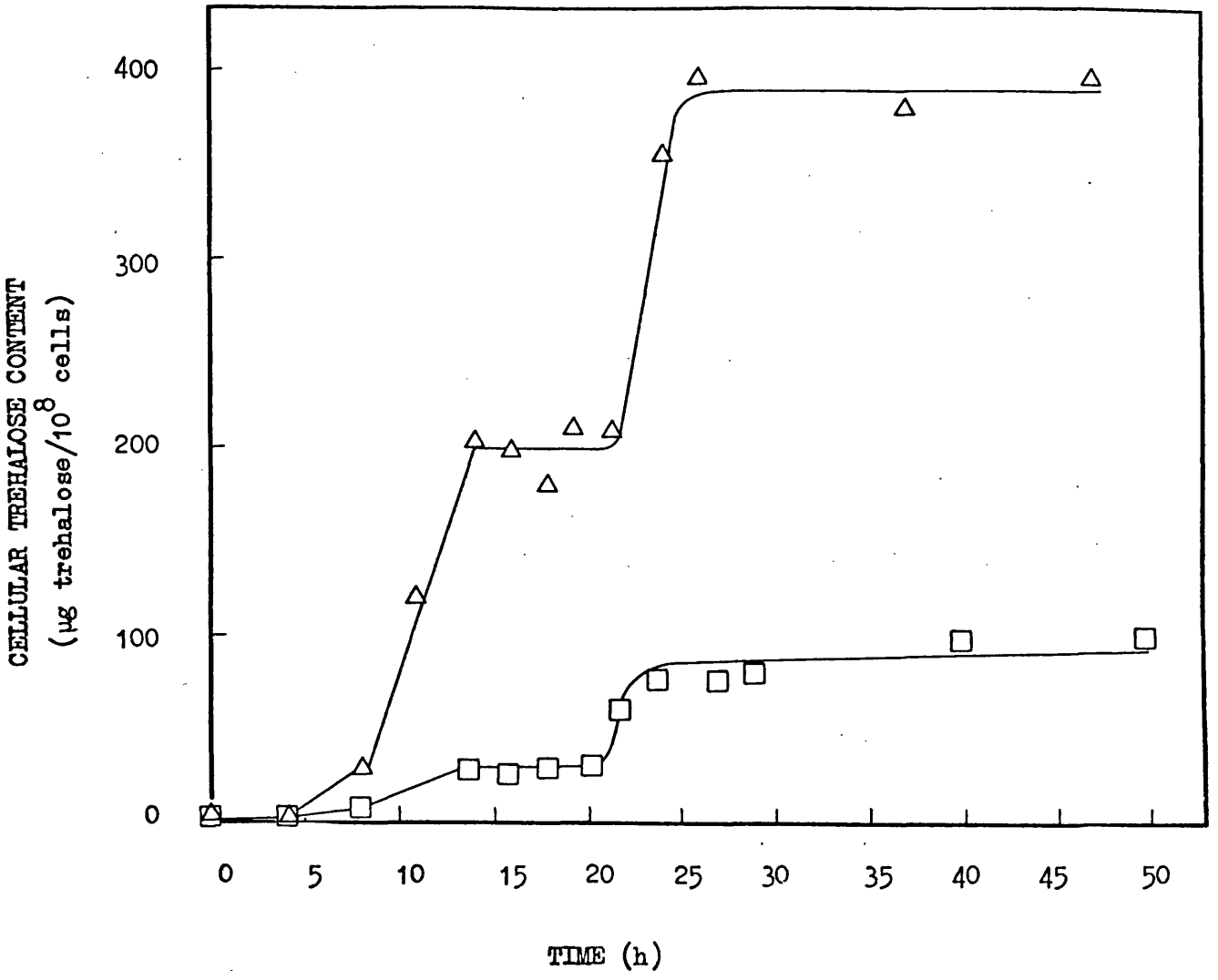


FIG. 19 Accumulation of Trehalose during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.31mg glycogen/10^8 cells

△ ; myxamoebae initially contained 5.65mg glycogen/10^8 cells

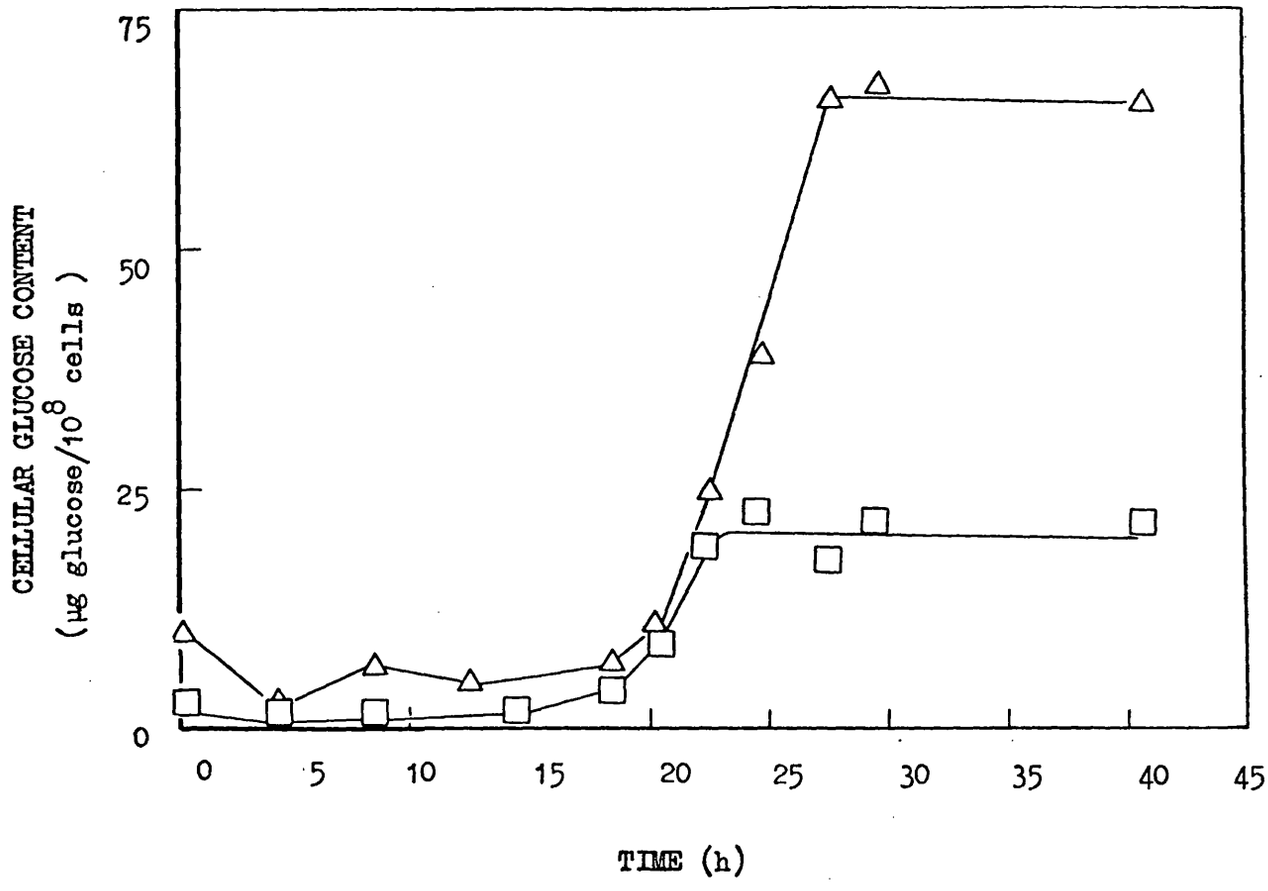


FIG. 20 Accumulation of Glucose during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.13mg glycogen/10^8 cells

△ ; myxamoebae initially contained 4.98mg glycogen/10^8 cells

TABLE 11 The relationship between myxamoebal glycogen content and the sorocarp content of end-product saccharides

*Myxamoebal glycogen content ($mg/10^8$ cells)	**Sorocarp mucopoly-saccharide content ($\mu g/10^8$ cells)	**Sorocarp cell wall polysaccharide content ($\mu g/10^8$ cells)	**Sorocarp trehalose content ($\mu g/10^8$ cells)	**Sorocarp glucose content ($\mu g/10^8$ cells)
0.070 ± 0.008 (4)	42.19 ± 1.22 (6) 37.53 ± 1.11 (4)	237.37 ± 3.38 (5) 234.00 ± 12.49 (3)	-	-
0.308 ± 0.002 (2)	-	-	100.40 ± 3.72 (3) 108.94 ± 1.48 (3)	21.10 ± 0.84 (3) 24.32 ± 0.27 (3)
1.947 ± 0.044 (3)	54.83 ± 6.42 (5)	191.34 ± 8.45 (5) 201.25 ± 12.37 (4)	-	-
2.670 (1)	-	204.39 ± 6.71 (5)	-	-
3.490 ± 0.130 (3)	56.76 ± 2.24 (5)	235.67 ± 12.33 (3)	-	-
5.060 ± 0.042 (3)	70.01 ± 2.86 (5)	-	378.95 ± 6.62 (3) 421.59 ± 22.14 (3)	66.48 ± 0.57 (2) 66.16 ± 3.72 (3)
5.590 ± 0.048 (5)	79.03 ± 2.77 (5) 85.24 ± 12.13 (6)	281 ± 8.98 (5) 290.95 ± 8.38 (4)	390.33 ± 6.37 (3)	60.26 ± 0.87 (3)

Results are given as means $\pm$ S.E.M.

* Each set of figures represents the mean glycogen content for several experiments with the number of experiments given in brackets.

**Each set of figures represents a separate experiment with the number of determinations per experiment given in brackets

in the amount of glucose accumulated by cells derived from myxamoebae containing high or low concentrations of glycogen initially; in Fig.20 and Table 11 we show the two extreme cases. Interestingly, the glucose pool decays only slowly after fruiting body formation.

The Control of Trehalose accumulation during Development

Trehalose accumulation was examined in preference to the accumulation of the other end-product saccharides since it showed the largest variation in response to changes in the level of vegetative myxamoebal glycogen, the synthetic pathway leading to trehalose is short and well known, and the rate limiting enzymic step well characterised (Roth & Sussman, 1966, 1968).

In order to elucidate the control mechanisms operating on trehalose accumulation during development, I have assayed the activities of the enzymes relevant to the process, and determined the pool sizes of important metabolic intermediates in myxamoebae accumulating markedly different amounts of trehalose.

1. Trehalase and Trehalose 6-phosphate (T6P) Synthase Activities during Development

Axenically-grown vegetative myxamoebae definitely possess both trehalose synthetic (T6P synthase) and trehalose degradative (trehalase) enzymes unlike bacterially-grown cells which are reported to lack T6P synthase activity by some workers (Roth & Sussman, 1968) but not others (Killick & Wright, 1972). The reason for this discrepancy may lie in the stability of T6P synthase; Killick & Wright

(1972) have recently shown that this enzyme is cold labile, activity decaying much more rapidly at 2°C than at 25°C. Now Roth & Sussman (1968) assayed T6P synthase in cell samples which had been frozen for several hours, whereas the data presented here and that of Killick & Wright (1972) was obtained using fresh cell extracts.

Although axenically-grown vegetative myxamoebae possess high activities of trehalase, the enzyme decays rapidly during aggregation, soon reaching a low level which is then maintained throughout the rest of development (Fig. 21). This pattern of activity is qualitatively similar to that reported to occur during the development of bacterially-grown cells (Ceccarini, 1967) although axenically-grown cells possess approximately 10-fold higher trehalase activity than bacterially-grown cells at all stages of development. Garrett & Sussman (1972) have reported that the trehalase of N. crassa probably exists in an active and an inactive form, activation occurring after freezing and thawing in 0.05 M phosphate buffer, pH 5.6. I have been unable to show any difference in trehalase activity between fresh and frozen extracts using my buffer system (Table 12). The high activity of trehalase in myxamoebal extracts is thus not artefactual.

The pattern of trehalase activity during the development of axenically-grown cells is unaffected either qualitatively or quantitatively by variations in the vegetative myxamoebal glycogen content (Fig. 21).

The pattern of T6P synthase activity during development is completely different from that of trehalase; axenically-grown myxamoebae contain only low specific activities of the enzyme and activity

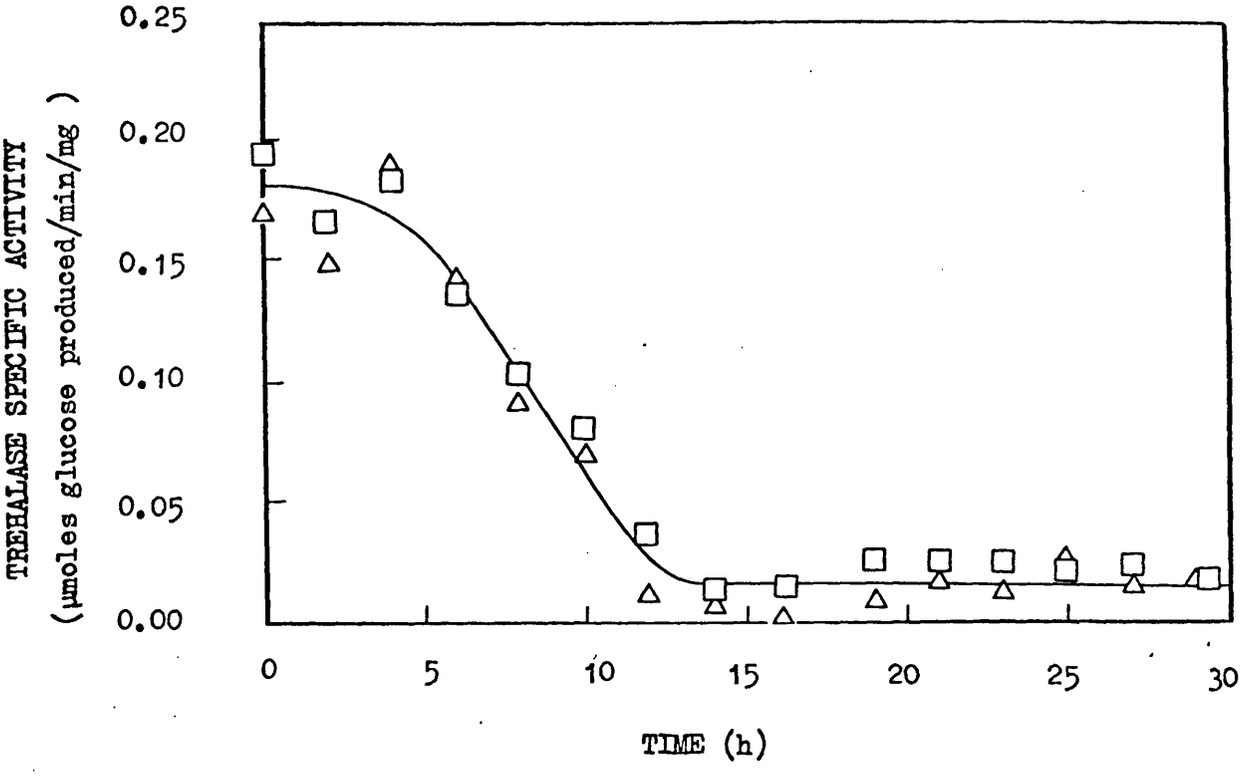


FIG.21 Variation in Trehalase Activity during The Development of Axenically-grown Myxamoebae.

□; myxamoebae initially contained 0.29mg glycogen/10^8 cells

△; myxamoebae initially contained 5.95mg glycogen/10^8 cells

TABLE 12 To show absence of trehalase activation upon freezing and thawing in 0.016 M phosphate buffer, pH 5.6

Sample	Trehalase activity (μmoles glucose produced/ min/mg)
* Unfrozen trehalase	0.129 ± 0.005 (2)
**Frozen trehalase	0.131 ± 0.008 (2)

Results given as means ± S.E.M.

Figures in brackets refer to the number of determinations

The myxamoebae used in this experiment initially contained 5.95 mg glycogen/10^8 cells.

* Cell sample ultrasonicated and assayed for trehalase activity immediately

** Cell sample frozen at -15°C for 48 h in 0.016 M phosphate buffer pH 5.6, thawed in the cold, ultrasonicated, and assayed for trehalase activity

increases during development, reaching a peak just before fruiting body construction (Fig. 22). A similar pattern of T6P synthase activity is observed during the development of bacterially-grown cells (Roth & Sussman, 1968). Whilst the peak of T6P synthase activity observed during the development of axenically-grown myxamoebae is often broader for cells initially containing high glycogen contents than for cells initially containing low glycogen contents, the peak specific activities observed have not differed by more than 25% (Fig. 22, Table 13a).

2. UDP-glucose and Glucose 6-phosphate Pools during Development

UDPG and G6P, the substrates for T6P synthase, have been reported to increase in cellular concentration during the development of bacterially-grown myxamoebae, only decreasing during fruiting body construction when end-product saccharide synthesis is occuring (Wright et al., 1964; Pannbacker, 1967).

During the development of axenically-grown myxamoebae, the UDPG pool varies in a qualitatively similar manner as described above for bacterially-grown myxamoebae (Figure 23). The G6P pool, however, reaches a steady state concentration after the first few hours of development, transiently increases at culmination, and finally decreases to a basal level (Fig. 24).

Both UDPG and G6P pools are influenced markedly by the vegetative myxamoebal glycogen contents. Thus midway during development, cells initially containing 6.58 ± 0.46 mg glycogen/10^8 cells possess UDPG and G6P pools three times the size of those in myxamoebae initially containing 0.24 ± 0.03 mg glycogen/10^8 cells (Figs. 23,24, Table 13a).

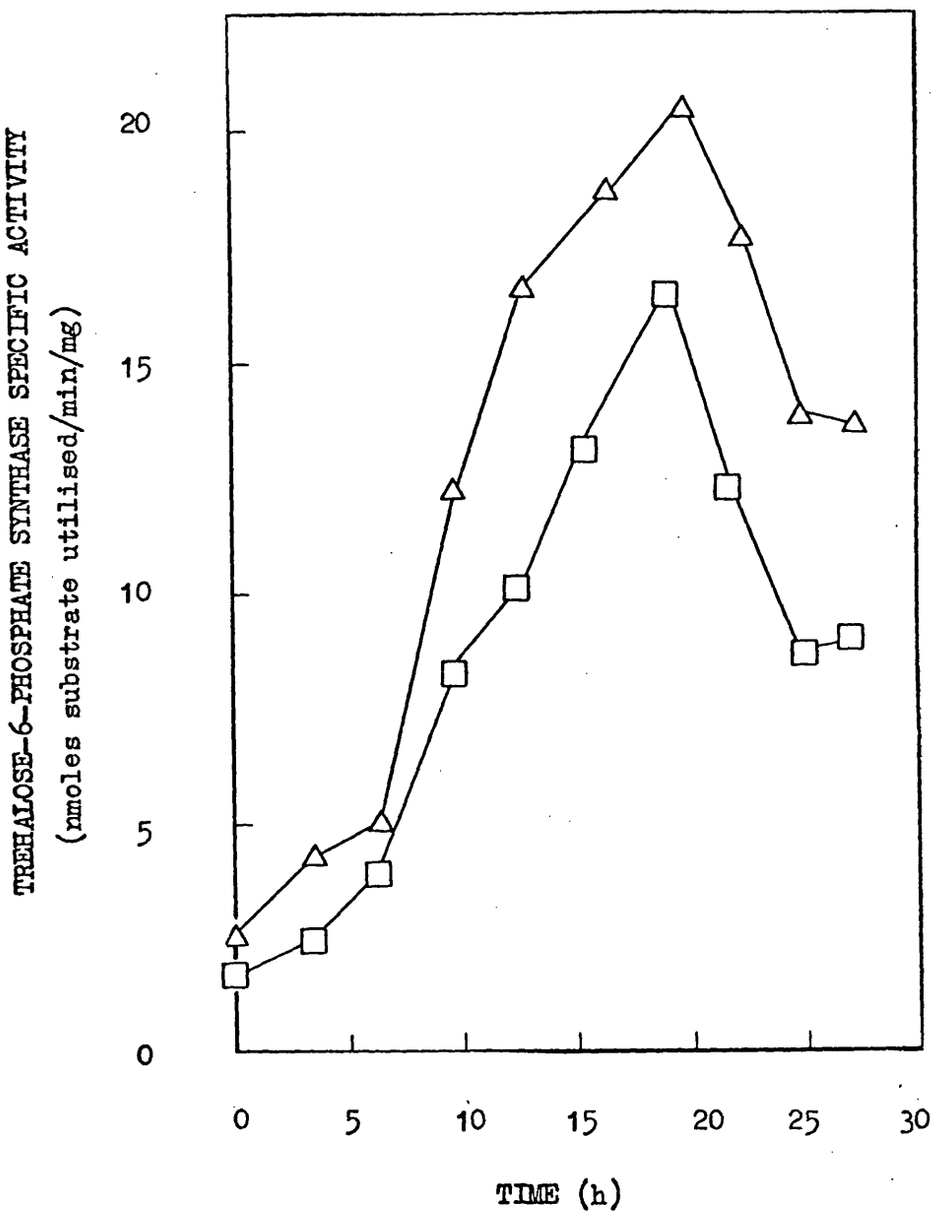


FIG. 22 Variation in Trehalose-6-Phosphate Synthase Activity during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.29mg glycogen/10^8 cells

△ ; myxamoebae initially contained 5.95mg glycogen/10^8 cells

TABLE 13 (a) The cellular content of UDPG, G6P, and T6P synthase, during the development of myxamoebae initially containing various amounts of glycogen

Myxamoebal glycogen content (mg/10^8 cells)	*Cellular G6P content (nmoles/10^8 cells)	*Cellular UDPG content (nmoles/10^8 cells)	Peak T6P synthase specific activity (nmoles substrate used/min/mg)
0.24 ± 0.03 (4)	3.37 ± 0.32 (3)	14.72 ± 2.44 (3)	16.21 ± 0.61 (2)
6.58 ± 0.46 (4)	11.93 ± 1.41 (3)	42.33 ± 5.69 (3)	20.21 ± 0.71 (2)

(b) The cellular concentrations of UDPG and G6P during the development of myxamoebae initially containing various amounts of glycogen

Myxamoebal glycogen content (mg/10^8 cells)	** Cellular G6P concentration (10^{-3} M)	Cellular UDPG concentration (10^{-3} M)
0.24 ± 0.03 (4)	0.068 ± 0.006 (3)	0.294 ± 0.049 (3)
6.58 ± 0.46 (4)	0.239 ± 0.028 (3)	0.846 ± 0.114 (3)

Results are given as means ± S.E.M. Figures in brackets refer to the number of experiments

* At 11 h developmental time

** At 11 h developmental time, assuming that the intracellular volume of 10^9 cells is 0.5 ml.

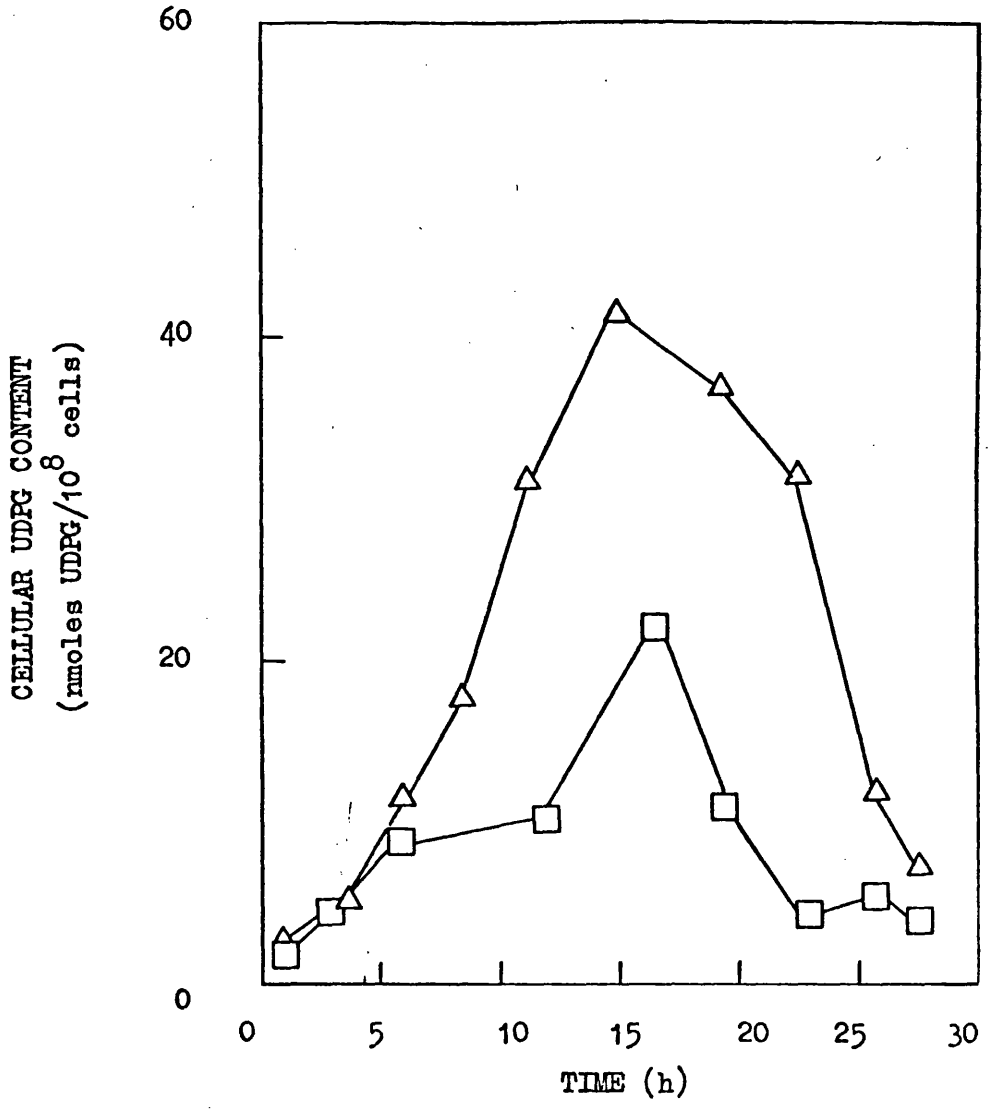


FIG.23 Changes in The Cellular Content of UDPG during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.26mg glycogen/10^8 cells

△ ; myxamoebae initially contained 7.80mg glycogen/10^8 cells

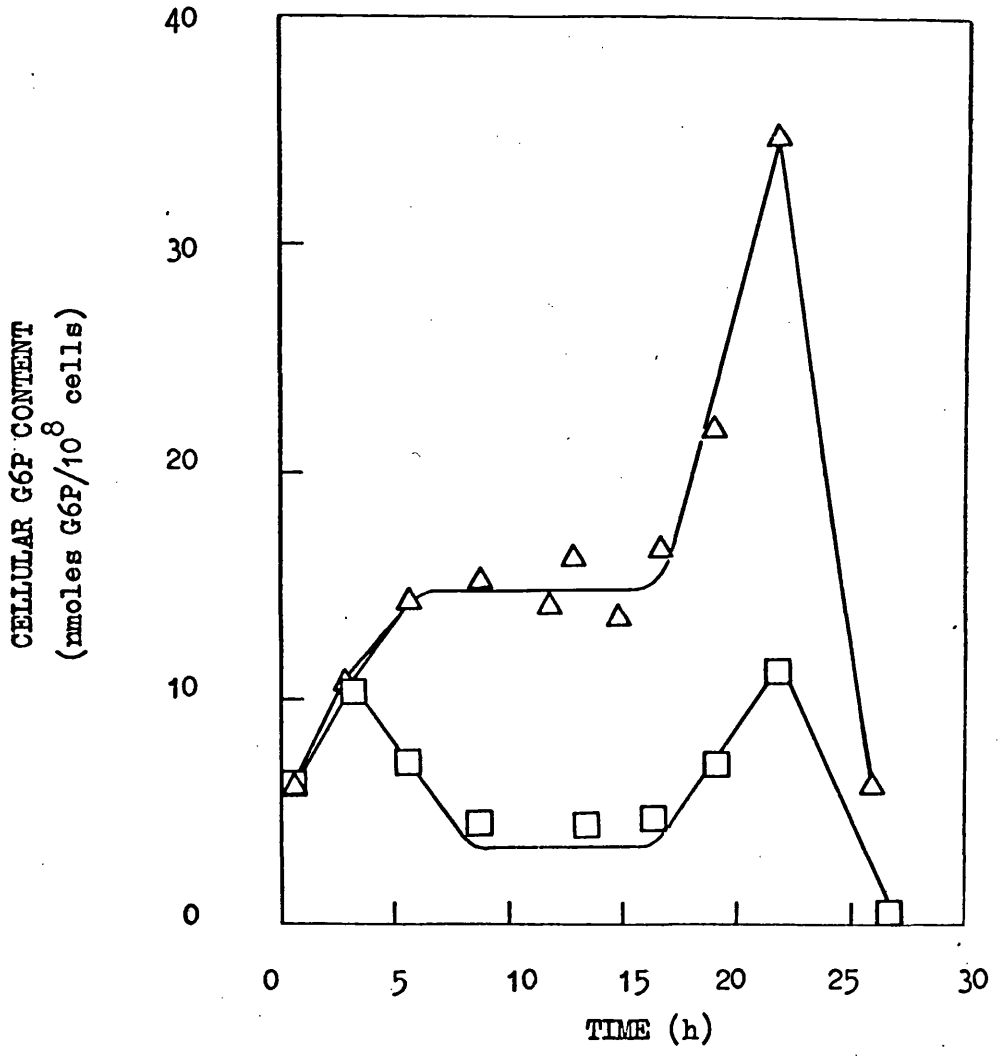


FIG. 24 Changes in The Cellular Content of G6P during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.26mg glycogen/10^8 cells

△ ; myxamoebae initially contained 7.80mg glycogen/10^8 cells

Assuming that the intracellular volume of 10^9 axenically-grown myxamoebae is approximately 0.5 ml, the maximum intracellular concentration of both UDPG and G6P during the development of cells initially containing 6.58 ± 0.46 mg glycogen/10^8 cells is approximately 8×10^{-4} M.

3. Kinetic Parameters of T6P synthase

In order to determine whether the changes in the intracellular concentration of UDPG and G6P would significantly affect the activity of T6P synthase, the K_m's of this enzyme for these substrates were determined (Figs. 25,26) and found to be 1.0×10^{-3} M (for UDPG) and 5.6×10^{-3} M (for G6P). Clearly, the variations in *in vivo* substrate concentrations observed (Table 13b) are of a magnitude to alter the activity of T6P synthase.

The K_m values obtained are in good agreement with those reported previously by Roth & Sussman (1968) using bacterially-grown cells.

FIG.25 Determination of The Michaelis Constant (Km) of Trehalose-6-Phosphate Synthase for UDPG.

Fig.25(a) Variation in Trehalose-6-Phosphate Synthase Activity with UDPG Concentration.

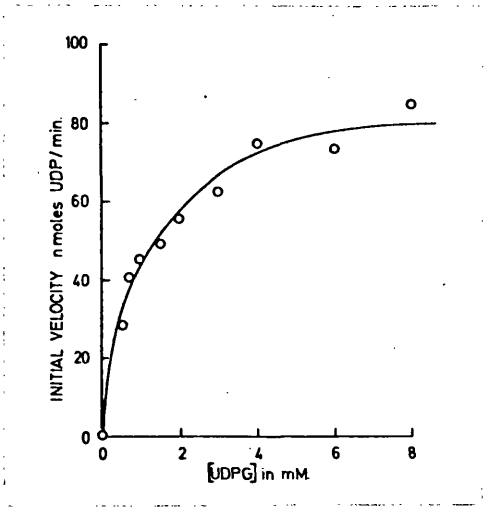


Fig.25(b) Lineweaver-Burk (1934) Plot of Variation in Trehalose-6-Phosphate Synthase Activity with UDPG Concentration.

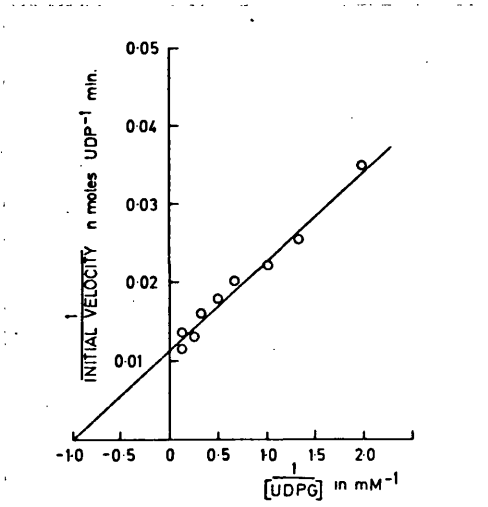


FIG.26 Determination of The Michaelis Constant (Km) of Trehalose-6-Phosphate Synthase for G6P.

Fig.26(a) Variation in Trehalose-6-Phosphate Synthase Activity with G6P Concentration.

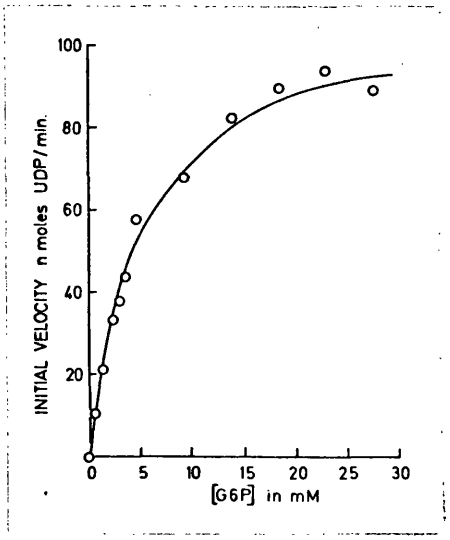
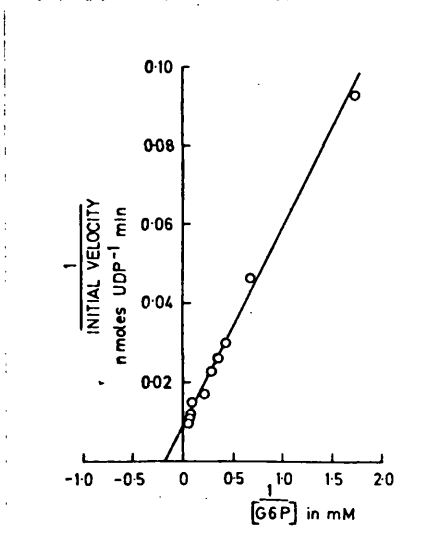


Fig.26(b) Lineweaver-Burk (1934) Plot of Variation in Trehalose-6-Phosphate Synthase Activity with G6P Concentration.



DISCUSSION

Probably more is known of carbohydrate metabolism than any other biochemical aspect of the developmental phase of D. discoideum. Characteristically, development in this organism results in the synthesis of three main carbohydrates not present to any large extent during vegetative growth and these (trehalose, cell wall polysaccharides, and an acid mucopolysaccharide) have been termed end-product saccharides (p. iv), since once synthesised they are not degraded during the developmental phase. In addition, glucose accumulates during fruiting body construction and the glucose pool size remains constant or decays only slowly in the mature sorocarp (Ceccarini & Filosa, 1965; White & Sussman, 1963a; this chapter) possibly serving as an energy source to maintain the low metabolic rate of dormant spores*, as well as being concerned with spore germination (Cotter & Raper, 1970). Glucose accumulation at culmination may therefore be just as important as the synthesis of end-product saccharides and so the term end-product saccharides as used in this discussion will include glucose, even though this compound is metabolised after fruiting body formation. If saccharides other than those described above are made and retained in the mature sorocarp, it is unlikely that they occur in large amounts since the total hexose content of mature sorocarps is approximately

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* Data shown in Table 14 indicate that the glucose content of sorocarps is localised almost entirely in the spores and hence the slow decrease in sorocarp glucose content which is sometimes observed must be due to its metabolism by the dormant spores.

TABLE 14 The distribution of glucose in fruiting bodies derived from the development of axenically-grown myxamoebae

Sample	Cellular glucose content ($\mu g/10^8$ myxamoebae)
Stalk cells	1.42 ± 0.12 (2)
Spores	57.73 ± 2.05 (2)

Results are given as means $\pm$ S.E.M.

Figures in brackets refer to the number of determinations

Comparison of the glucose content of stalk cells and spores on the basis of cell number is not feasible since the stalk cells are encased in the stalk matrix rendering them impossible to count. Likewise, protein content has not been used as a basis because the stalk cells are known to metabolise more of their protein during development than spore cells (Gregg *et al.*, 1954). Thus, comparison has been made on the basis of myxamoebal number; for example, the stalk cell glucose content of 1.42 ± 0.12 (2) $\mu g/10^8$ myxamoebae should be interpreted as the glucose content of all the stalk cells produced by the development of 10^8 myxamoebae.

equal to the sum of the glycogen, glucose, trehalose, cell wall polysaccharide and mucopolysaccharide hexose contents (Table 15a,b).

The Control of End-Product Saccharide Synthesis - A General View

It is generally believed that glycogen acts as the precursor for end-product saccharide synthesis although this has not been adequately proved. However, the peak cellular content of developmental glycogen during the development of myxamoebae grown in axenic medium lacking added glucose is only 35% of the total end-product saccharide + residual glycogen hexose present in the mature sorocarp (Table 16). Also, the total hexose content of the cells continues to rise after developmental glycogen degradation has begun (Fig. 27). One must therefore conclude that either developmental glycogen is the sole source of hexose for end-product saccharide synthesis in these cells and therefore degradation and utilisation of developmental glycogen for this purpose begins while developmental glycogen synthesis is still occurring, or, synthesis of end-product saccharides may also (or solely) use hexose derived via gluconeogenesis but which never enters the developmental glycogen pool (e.g. UDPG, G6P). It is interesting that whilst two of the end-product saccharides synthesised during fruiting body construction (trehalose and mucopolysaccharide) have been tentatively identified as being synthesised in vesicles (see later), there is a marked lack of vesicular elements in the regions of active stalk cell wall synthesis which occurs at or very near the cell membrane itself (George et al., 1972). Now, electron microscopical studies have revealed that the spore coat consists of three layers, the innermost

TABLE 15 Comparison of the sorocarp content of known carbohydrates and of anthrone-positive material

(a) Sorocarps derived from the development of axenically-grown myxamoebae initially containing less than 0.32 mg glycogen/10^8 cells

Sorocarp carbohydrate content ($\mu g/10^8$ cells)		Sorocarp content of anthrone-positive material (μg glucose equivs./10^8 cells)
Cell wall polysaccharide	* 240	
Trehalose	**100	
Mucopolysaccharide	* 40	
Glucose	** 20	
Glycogen	* 60	
Total	460	*520

* Myxamoebae initially contained 0.071 $\pm$ 0.008(4) mg glycogen/10^8 cells
**Myxamoebae initially contained 0.308 $\pm$ 0.002(2) mg glycogen/10^8 cells
Results given as means $\pm$ S.E.M.; figures in brackets refer to the number of experiments

(b) Sorocarps derived from the development of bacterially-grown myxamoebae (Data taken from Sussman & Sussman, 1969).

Sorocarp carbohydrate content ($\mu g/10^8$ cells)		Sorocarp content of anthrone-positive material (μg/glucose equivs./10^8 cells)
Cell wall polysaccharide	90	
Trehalose	140	
Mucopolysaccharide	50	
Glucose	15	
Glycogen	20	
Total	315	410

NOTE: The anthrone reagent is also known to react with RNA and protein as well as with carbohydrates (Colowick & Kaplan, 1957) and thus one might expect the cellular content of anthrone-positive material to exceed the cellular content of carbohydrate

TABLE 16 Comparison of the sorocarp content of carbohydrate synthesised during culmination and the peak cellular content of developmental glycogen

	I	II	III
	Sorocarp content of carbohydrate synthesised during culmination	Sorocarp content of carbohydrate synthesised during culmination minus cell wall polysaccharide	Peak cellular content of developmental glycogen
$\mu g/10^8$ cells	390*	180**	135 ± 10 (2)
Percentage values	100.0	46.2	34.6

Figures are given as means $\pm$ S.E.M. with the number of experiments given in brackets

* This figure is derived from Table 14 and Figs. 18, 19 by subtraction of the pre-culmination cellular trehalose and alkali-insoluble (cell wall polysaccharide?) contents, i.e. 460 minus about 70 = 390 $\mu g/10^8$ cells.

**This figure is derived from the figure in column I by subtraction of the sorocarp content of cell wall polysaccharide actually synthesised during culmination (see Fig. 18 , Table 14), i.e. 390 minus about 210 = 180 $\mu g/10^8$ cells.

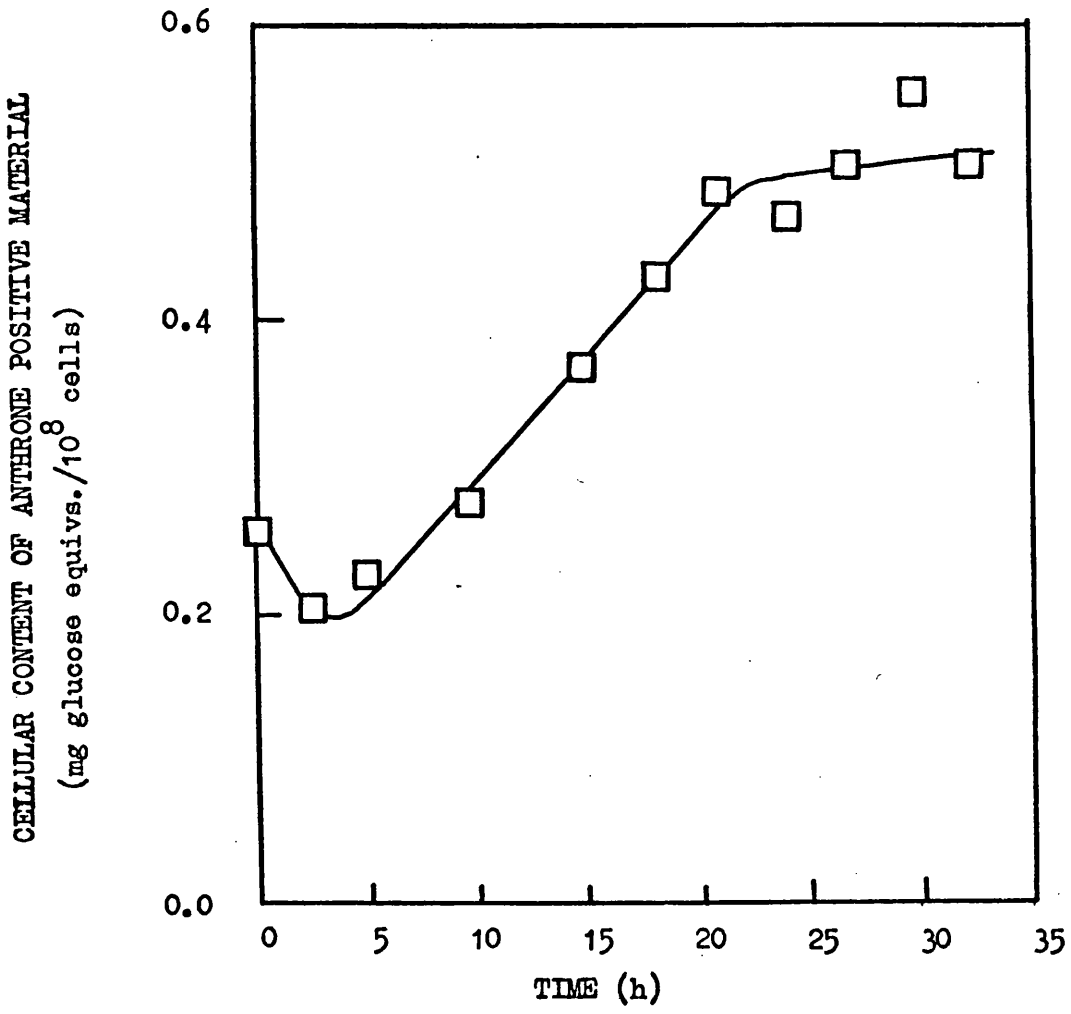


FIG.27 Changes in The Cellular Content of Anthrone positive Material during The Development of Axenically-grown Myxamoebae.
The myxamoebae initially contained 0.074mg glycogen/10^8 cells.

two of which appear to comprise the cell wall polysaccharide; it has been suggested that the thickest central layer is composed of glycogen (Cotter et al., 1969; Hohl & Hamamoto, 1969). One wonders whether cell wall synthesis uses as precursor the glycogen component of cell wall polysaccharide, synthesised by the glycogen synthetase previously responsible for developmental glycogen synthesis but now located at the cell wall site. If one assumes this to be so, the amount of alkali-soluble developmental glycogen formed during development is probably quite sufficient to account for the amounts of the other end-product saccharides which accumulate during fruiting body construction (Table 16). Whichever scheme is correct, it is a fact that the end-product saccharides are synthesised almost entirely using hexose derived via gluconeogenesis (Chapter II). The exception is myxamoebae which contain large reserves of myxamoebal glycogen as a result of growth in axenic medium containing added glucose. Under these conditions, some of the hexose for end-product saccharide synthesis comes from myxamoebal glycogen degradation (cells containing high levels of myxamoebal glycogen, labelled with [U-^{14}C]glucose, are found to transfer a significant fraction of the label to end-product saccharides synthesised during the developmental phase (Chapter II, Table 17)) although some hexose is still derived from non-glycogen sources (as shown by the marked lowering in specific radioactivity of end-product saccharide carbon compared to myxamoebal glycogen carbon (Table 17). Whatever the ultimate source, the immediate precursor for all end-product saccharide synthesis so far studied (except possibly cell wall cellulose synthesis - see later) is UDPG (Roth & Sussman, 1966; Ward & Wright, 1965; Newell & Sussman, 1969) although trehalose synthesis

TABLE 17 The use of myxamoebal glycogen as precursor for end-product saccharide synthesis

Compound	Specific Radioactivity (cpm/μg atom carbon)	
	Experiment I	Experiment II
Cellular glycogen		
0 h	1,820	1,806
40 h	-	1,910
Cellular protein		
0 h	188	257
40 h	-	274
Cell wall polysaccharide	1,120	1,110
Trehalose	-	927
Glucose	-	1,251

Experiment I : Myxamoebae initially contained 6.10 mg glycogen/10^8 cells

Experiment II : Myxamoebae initially contained 5.25 mg glycogen/10^8 cells

also requires G6P (Roth & Sussman, 1966).

Except for trehalose synthesis (Roth & Sussman, 1966, 1968), the pathways and enzymes of end-product saccharide synthesis are little characterised and hence little is known of control mechanisms. Obviously there is control over the initiation of synthesis since end-product saccharides are synthesised only at specific times in the developmental phase, but are there controls governing the amounts of end-product saccharides synthesised?

Unfortunately, myxamoebae grown on bacteria have been used for all studies of end-product saccharide synthesis prior to that described here. Since one would expect these cells always to have more or less the same chemical composition, it is not too surprising that they always make approximately the same amount of end-product saccharide during development. Thus, bacterially-grown myxamoebae always synthesise approximately 90 μg cell wall polysaccharide, 50 μg mucopolysaccharide, 140 μg trehalose, and 15 μg glucose per 10^8 cells (Sussman & Sussman, 1969 ; White & Sussman, 1961). Certainly, not as much mucopolysaccharide is made as trehalose, for example, but this could reflect the inherently different activities of the biosynthetic enzymes of the various pathways. The enzymes would then utilise the substrates available until these were exhausted. Given that the chemical composition of myxamoebae grown on bacteria is largely invariant, the myxamoebal substrate pool size would not vary, and one would observe an unchanging pattern of end-product saccharide synthesis. This data therefore really gives no information on the nature of the controls which terminate end-product saccharide synthesis.

In order to determine whether there are controls, other than substrate availability, which limit end-product saccharide synthesis, and exactly what those controls are, one really needs a system in which experimental alteration of the amounts of end-product saccharides synthesised is possible.

The results presented in this chapter show that the development of axenically-grown myxamoebae containing various levels of myxamoebal glycogen is such a system. The amounts of trehalose, cell wall polysaccharide, mucopolysaccharide, and glucose which are synthesised during development are a direct function of the myxamoebal glycogen content; myxamoebae initially containing 5.59 ± 0.048 mg glycogen/10^8 cells synthesise up to 30% more cell wall polysaccharide, 200% more mucopolysaccharide, 300% more glucose and 400% more trehalose than myxamoebae initially containing less than about 0.32 mg glycogen/10^8 cells (Table 11). Important in this respect is that the pool sizes of UDPG and G6P, the substrates for end-product saccharide synthesis are much larger throughout the development of the former compared to the latter cells.

These data, considered in conjunction with the results gained from studies of the development of bacterially-grown myxamoebae, help to define the controls regulating end-product saccharide synthesis.

Firstly, it is obvious that the pool of material which is the precursor for end-product saccharide synthesis is not closed; even though gluconeogenesis and developmental glycogen may provide the hexose units for end-product saccharide synthesis, glucose liberated by myxamoebal glycogen degradation may also be used for this purpose. The only proviso is that since the products of myxamoebal glycogen degradation

are usually rapidly oxidised to carbon dioxide (Chapter II) sufficient myxamoebal glycogen must be present in vegetative myxamoebae so that myxamoebal glycogen degradation is still occurring at or just before the time of end-product saccharide synthesis. Apparently only under these conditions can end-product saccharide synthesis compete with oxidative processes for the hexose liberated by myxamoebal glycogen degradation. Theoretically, this alone is not sufficient to cause an increase in the sorocarp content of end-product saccharides since stringent regulation of end-product saccharide synthesis could ensure the production of a fixed amount of end-product saccharide irrespective of substrate pool size. The observation that the fruiting body content of trehalose, mucopolysaccharide, and glucose (but not cell wall polysaccharide) can be increased, but that the percentage increase is specific for each saccharide, rather than the synthesis of each saccharide being increased proportionately, indicates that:

(1) Controls exist which determine the maximum amount of each end-product saccharide made,

(2) for trehalose, mucopolysaccharide, and glucose, the amount of end product saccharide synthesised is not absolutely fixed but is subject to change depending on cellular conditions,

(3) for cell wall polysaccharide, synthesis is tightly controlled to ensure an invariant sorocarp cellulose content under conditions when the sorocarp content of the other end-product saccharides varies greatly,

(4) the controls which determine the quantity of each end-product

saccharide synthesised are not coordinate,

(5) large variations in the amounts and proportions of end-product saccharides synthesised do not impair the ability of D. discoideum to develop normally and produce normal fruiting bodies.

Perhaps the most important conclusion is that the controls which operate to determine the amount of end-product saccharides formed during development are not coordinate. Since trehalose, mucopolysaccharide, and cell wall polysaccharide synthesis all require UDPG as substrate, a simple way to ensure the formation of a sorocarp of invariant composition would be for these syntheses to use UDPG from a common pool. The amount of each end-product saccharide made would depend on the activity of the synthetic enzymes of that pathway but once the amount of any one end-product saccharide reached a critical level it would inhibit UDPG pyrophosphorylase, the enzyme synthesising UDPG, and hence inhibit the synthesis of all these end-product saccharides. Indeed, Gustafson & Wright (1971) have reported that UDPG pyrophosphorylase is inhibited during fruiting body construction.

A control mechanism of this type would therefore ensure that the end-product saccharides of D. discoideum were always synthesised in strict proportion to one another. However, the fact that the amounts of end-product saccharides present in the mature sorocarp can vary independently of one another indicates that in this case each pathway must have its own individual control.

Now, Newell & Sussman (1969) reported that UDPG pyrophosphorylase is polymorphic and have proposed that each end-product

saccharide may be synthesised in a separate location with its own UDPG pyrophosphorylase and presumably its own pool of UDPG. According to this scheme, not only could the rate of synthesis of any one end-product saccharide be governed by the activity of the attendant UDPG pyrophosphorylase (provided it is rate limiting) but termination of synthesis after a certain amount of end-product had been synthesised could be achieved by inhibition of the corresponding UDPG pyrophosphorylase, without affecting the synthesis of the other end-product saccharides. Although a scheme of this kind could account for the results presented in this chapter, Pannbacker (1967b) finds no evidence for the compartmentalisation of UDPG pyrophosphorylase; whether extracted by freezing or gentle homogenisation, the activity is completely soluble when spun at 30,000 g for 30 min.

The only conclusion must be that the control of end-product saccharide synthesis is specific for each saccharide and that the control operates after UDPG formation. Therefore, let us examine the synthesis of each end-product saccharide in turn using both the evidence presented in this chapter and that derived from studies using bacterially-grown cells to attempt to understand how the developing cell regulates the amount of saccharide formed.

There must be three controls which operate on the synthesis of any end-product saccharide:

(1) Control of the initiation of synthesis

(2) Control of the rate of synthesis

(3) Control of the termination of synthesis.

Initiation of end-product saccharide synthesis occurs at the

same time in development irrespective of the amount of end-product saccharide made. Therefore, only data concerning control of the rate or termination of end-product saccharide synthesis will be considered in detail here.

The control of Cell Wall Polysaccharide Synthesis

Raper & Fennel (1952), Mühlethaler (1956) and Gezelius & Ranby (1957) showed the presence of a cellulose-like polymer in the cell wall of D. discoideum but it was not until 1965 that Ward & Wright demonstrated that the cell wall is really a complex of cellulose-like and glycogen-like polymers. Ward & Wright (1965) and Wright & Dahlberg (1967) investigated the synthesis of cell wall but found that in vitro preferential synthesis of the glycogen-like polymer (cell wall glycogen) occurs. The amount of the cellulose-like polymer (cell wall cellulose) synthesised under these conditions is not only low but also very variable, and consequently there is no data available concerning either the mechanism or control of cell wall cellulose synthesis in D. discoideum.

As previously described, cells containing high levels of myxamoebal glycogen synthesise much increased amounts of all end-product saccharides except cell wall polysaccharide, even though every one of these syntheses appears to use UDPG as substrate, pointing to a very stringent control of cell wall synthesis. How is this control achieved?

Although in vitro cell wall glycogen is synthesised in preference to cell wall cellulose, in vivo these compounds always

occur in equal proportions (by weight) (Ward & Wright, 1965) suggesting that their synthesis may be coordinately regulated. If so, the extent of cell wall synthesis could be determined by controlling the synthesis of either cell wall glycogen or cell wall cellulose alone.

Let us consider each in turn. Cell wall glycogen is synthesised by the same glycogen synthetase responsible for synthesising cytoplasmic glycogen except that near the end of development the enzyme is transferred from its cytoplasmic site of glycogen synthesis to a cell wall site and continues to make glycogen which is now incorporated into the cell wall (Wright et al., 1968a). The enzyme used UDPG as substrate and is stimulated by G6P but not (reproducibly) by cell wall polysaccharide itself (as acceptor). The extent of G6P stimulation depends not only on the concentration of G6P but also on the concentration of UDPG present, maximum activation occurring at high G6P concentrations (10^{-3} M) and low UDPG concentrations (10^{-4} M) and decreasing as the UDPG concentration rises or the G6P concentration falls (Wright, 1965). As pointed out by Wright (1965) this is probably a mechanism for buffering the rate of cell wall synthesis against variations in the cellular UDPG pool size. However, it is not possible to state with certainty that this phenomenon is sufficient to account for the invariant amount of cell wall polysaccharide synthesised during the development of axenically-grown cells containing different amounts of myxamoebal glycogen and possessing markedly different cellular UDPG and G6P pool sizes. The reason for this is that cellular UDPG and G6P concentrations, whatever

their value, vary with respect to each other and with time during the period of cell wall synthesis (Figs. 23, 24) and thus the extent of G6P activation of cell wall glycogen synthetase will also vary with time.

Are there any other controls which could prevent an increase in the amount of cell wall synthesised under conditions of increased cellular UDPG and G6P levels?

There are two other possible controls of cell wall synthesis exerted via control of cell wall glycogen synthesis. Either cell wall glycogen synthetase is working at maximum velocity such that increases in the substrate pool fail to result in increases in the amount of product made, or cell wall glycogen synthetase activity may be in excess but cell wall synthesis is terminated after the formation of a specific amount of cell wall polysaccharide as a result of inhibition of enzyme activity by cell wall itself (end-product inhibition). In fact, neither of these controls operate *in vivo* since the K_m of cell wall glycogen synthetase for UDPG is 1.3×10^{-3}M (Ward & Wright, 1965) whilst the highest concentration of UDPG observed during the development of axenically-grown cells is less than 10^{-3}M, and secondly, cell wall polysaccharide fails to inhibit the enzyme (Ward & Wright, 1965).

However, the extent of cell wall synthesis could be determined by control of cell wall cellulose synthesis. Unfortunately, there are no studies of the cellulose biosynthetic system in *D. discoideum*, and indeed cellulose biosynthesis is little understood in any organism. For example, hexose phosphates (Hestrin, 1960), sugar nucleotides

Elbein et al., 1964; Barber et al., 1969; Glaser, 1958), and a glycolipid (Colvin, 1959; Khan & Colvin, 1961) have all been suggested as precursors of cellulose. Recently, Manley et al. (1971) using Acetobacter xylinum, reported the existence of an ethanol-soluble, branched, lipid acceptor which greatly stimulates both the rate and extent of cellulose synthesis, and tentatively identified G1P as the glucosyl donor. In this organism the lipid acceptor appears to be the limiting substrate for cellulose synthesis. One wonders whether a similar system could operate in D. discoideum. Wright may have been unable to obtain reasonable rates of cellulose synthesis either through use of the wrong glucosyl donor (G1P was not tested) or lack of the appropriate acceptor. Could this explain the rigid control on the amount of cell wall synthesised in conditions of varying UDPG and G6P concentration? It is known that developing cells of D. discoideum possess an active phosphoglucomutase (D.J. Watts, personal communication; Cleland & Coe, 1968) which will equilibrate G6P and G1P pools. Also there is evidence that UDPG pyrophosphorylase is inhibited at culmination (Gustafson & Wright, 1971) so that the flow of G1P to UDPG will be reduced. Thus one would expect the G1P pool to vary in concentration in accordance with the G6P pool and indeed Pannbacker (1967b) has shown this to be the case. Therefore, in conditions of elevated G6P levels, the cellular G1P concentration should also be elevated resulting in an increased rate of cell wall cellulose and hence cell wall synthesis. So, if G1P is the substrate for cell wall cellulose synthesis, control of cell wall synthesis cannot solely be via G1P concentration. ADPG, GDPG, and CDPG were

all tested by Ward & Wright (1965) and found to be poorer glucosyl donors for cell wall synthesis than UDPG. The only other likely glucosyl donor is glucose itself and this is known to be present in increased concentration when UDPG and G6P levels are elevated (Chapter II) but when the amount of cell wall synthesised is constant. Thus, it is unlikely that cell wall cellulose synthesis, and hence cell wall synthesis, is controlled solely by availability of the glucosyl donor.

Other possible control mechanisms are:

(1) cell wall cellulose synthetase is working at maximum velocity such that increases in the substrate pool fail to cause an increase in the amount of cell wall cellulose and hence cell wall synthesised,

(2) the extent of cell wall synthesis is limited by the availability of acceptors for cell wall cellulose synthesis,

(3) the rate of cell wall cellulose synthesis is constant, irrespective of fluctuations in the substrate pool size, as a result of allosteric modification of the type reported for cell wall glycogen synthetase (Wright, 1965),

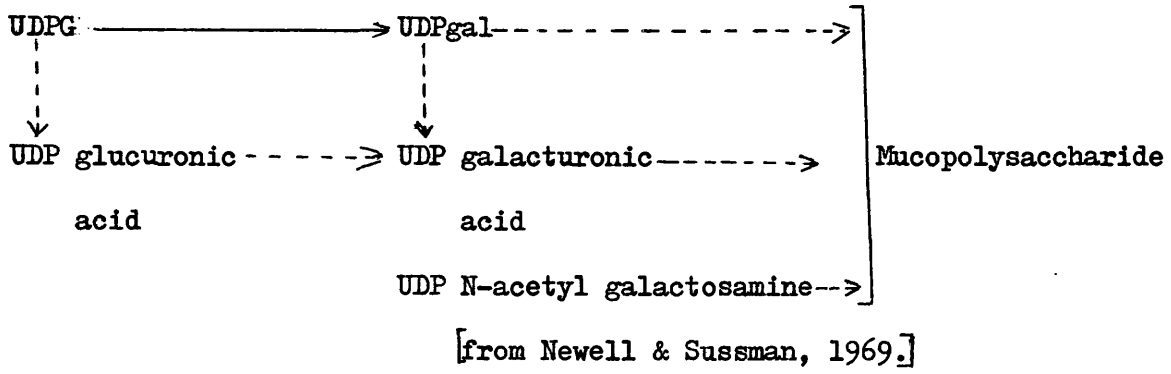
(4) cell wall cellulose synthetase is susceptible to end-product inhibition by cell wall.

It is clear that until the pathway of cell wall cellulose synthesis has been fully characterised, the mechanism of the control of cell wall synthesis will remain uncertain.

The Control of Mucopolysaccharide Synthesis

The pathway of mucopolysaccharide synthesis is not at all well

characterised, but is believed to be as shown below.



[from Newell & Sussman, 1969.]

Sussman & Osborne (1964) and Telser & Sussman (1971) have shown that two of the enzymes probably involved in the synthesis of mucopolysaccharide (UDPgal:polysaccharide transferase and UDPgal-4-epimerase respectively) are synthesised de novo during development. Moreover, galactose incorporation catalysed by UDPgal : polysaccharide transferase is stimulated greater than ten-fold by the presence of an ethanol-insoluble polysaccharide acceptor isolated from mature sorocarps. Interestingly, acceptor isolated from cells harvested at a time when the mucopolysaccharide content is only approximately 50% of the maximum level had 3 or 4-fold higher acceptor activity than that isolated from mature sorocarps. The composition of the two fractions is qualitatively similar but the latter has considerably higher galactosamine content suggesting to the authors that " in the intact cell the signal for the cessation of galactose incorporation into the polysaccharide is provided by the addition of N-acetyl galactosamine into terminal positions" * (Sussman & Osborne, 1964). So the extent of

* It is really rather surprising that termination of mucopolysaccharide synthesis occurs by acceptor inactivation since mucopolysaccharide is believed to be synthesised in spore specific vesicles which release their contents (mucopolysaccharide, certain synthetic enzymes and probably other materials) to the spore exterior during fruiting body construction (Höhl & Hamamoto, 1969; Tesler & Sussman,1971). One might expect after expulsion that, even if the synthetic enzymes remain in contact with active acceptor, synthesis would cease through lack of UDPG and that therefore there would be no need for a specific acceptor inactivation mechanism.

mucopolysaccharide synthesis is controlled by the availability of polysaccharide acceptor, synthesis terminating when the acceptor is inactivated. However, in the presence of increased levels of the other substrate, UDPG, the amount of mucopolysaccharide which is synthesised is doubled. Therefore termination of mucopolysaccharide synthesis does not occur after a certain amount of mucopolysaccharide has been formed, exempting end-product inhibition as the control mechanism. This leaves two broad categories of possible mechanisms for controlling the extent of mucopolysaccharide synthesis:

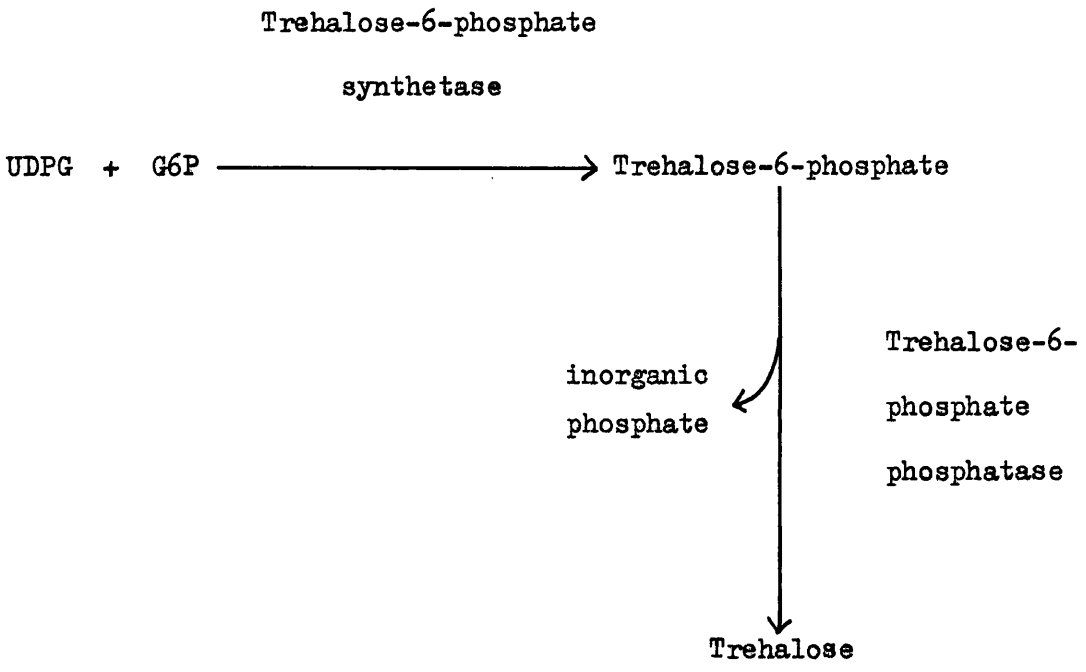
Firstly, termination of mucopolysaccharide synthesis could occur when a polysaccharide acceptor has reached a certain size. Increases in the amount of mucopolysaccharide made would then depend upon an increase in the number of acceptors available.

Secondly, inactivation of acceptor and hence termination could occur at a specific time in the developmental phase. Variation in the amount of mucopolysaccharide made would then be caused by alterations in the rate of synthesis by any of a number of mechanisms including variations in availability of substrates (UDPG, acceptor, etc.), variations in the cellular content of the rate limiting enzyme of the pathway, and allosteric modification of enzyme activity. (The correlation between the cellular pool size of UDPG and the amount of mucopolysaccharide synthesised is evidence in favour of the availability of this substrate controlling the rate and extent of mucopolysaccharide synthesis but does not exclude other mechanisms).

In summary, the lack of knowledge of the exact mechanisms of mucopolysaccharide synthesis and especially of the rate limiting enzyme of this synthetic pathway makes any hypothesis of control highly speculative.

From the preceding discussion it must be clear that our understanding of the control of cell wall and mucopolysaccharide synthesis is very poor, mainly because we lack knowledge of the exact mechanisms by which these polymers are synthesised.

However, this is not true of trehalose synthesis. The pathway is well characterised (Roth & Sussman, 1966, 1968) :-



No complex acceptors are involved, the synthesis really consisting of a condensation of two glucose molecules. Since trehalose 6-phosphate (T6P) has never been observed to accumulate during development (Roth & Sussman, 1966; Sargent & Wright, 1971), trehalose 6-phosphate synthetase (T6P synthetase) is considered the rate limiting enzyme.

The trehalose synthetic pathway was therefore chosen in preference to the other end-product saccharide synthetic pathways for a more detailed study of synthetic control because the pathway is short and well known, the rate limiting enzyme step well characterised (Roth & Sussman, 1966, 1968), and trehalose synthesis showed the largest variation in response to changes in the level of myxamoebal glycogen.

Control of Trehalose Synthesis

Initiation of trehalose synthesis always occurs at approximately the same time during the development of axenically-grown myxamoebae irrespective of the quantity of trehalose made.

The rate limiting enzyme for trehalose synthesis, T6P synthase, is not inhibited by as much as 40 mM trehalose (Roth & Sussman, 1966); approximately the same concentration as observed in spores produced from myxamoebae initially containing 5.590 ± 0.048 mg glycogen/10^8 cells. G6P has been found by some workers (Roth & Sussman, 1968) but not others (this chapter) to inhibit T6P synthetase at very high ($>3 \times 10^{-2}$ M) unphysiological concentrations, but this cannot limit the amount of trehalose synthesised since the cellular G6P concentrations,

is falling at the time of fruiting body construction. Also, the cells still contain high T6P synthetase activity at the time when trehalose synthesis ceases. Thus, there appears to be no specific mechanism to terminate trehalose synthesis other than depletion of the substrates, UDPG and G6P, which always occurs during sorocarp formation irrespective of their concentration.

Since initiation and termination of trehalose synthesis always occur at specific times in the developmental phase, the amount of trehalose synthesised will depend on the rate of trehalose synthesis which in turn will depend on the activity of T6P synthetase.

There are three ways to alter the activity of an enzyme:

(1) by variation of the amount of enzyme present,

(2) by variation of the concentration of substrates,

(3) by modification of enzyme activity via allosteric effectors and/or enzyme interconversions.

No allosteric effectors of the T6P synthetase of D. discoideum are known (except G6P; see above) even though they do exist in other systems (e.g. Sacktor, 1970) and only one form of T6P synthase has been reported, suggesting that the rate of trehalose synthesis is probably controlled either by the cellular content of T6P synthetase or by the cellular concentrations of UDPG and G6P, the enzymic substrates. However, myxamoebae of D. discoideum are known to also contain trehalase (Ceccarini, 1965, 1967; this chapter) which will degrade trehalose to glucose, and so increased trehalose accumulation could be caused by decreased trehalase activity, but since no allosteric effectors of trehalase are known, and trehalose is itself the substrate,

increased trehalose accumulation could only be caused by decreased trehalase activity. Therefore, in order to understand the control of trehalose synthesis, one must ask the question, is the rate of trehalose synthesis controlled by the cellular content of T6P synthetase, the cellular content of trehalase, or by the availability of the substrates for T6P synthetase (UDPG and G6P)?

The development of axenically-grown myxamoebae accumulating different amounts of trehalose provides an ideal system for investigations of this type.

The data presented in this chapter show that vegetative myxamoebae grown in axenic medium contain high trehalase activity which is retained during the first few hours of development but decays during aggregation to a low level that is then maintained throughout the rest of development. In contrast, these vegetative myxamoebae contain only a low specific activity of T6P synthetase but this greatly increases during development, reaching a peak just prior to fruiting body construction, and then decaying through fruiting body formation.

Now, axenically-grown myxamoebae containing 5.590 ± 0.048 mg glycogen/10^8 cells synthesise four times as much trehalose during development as myxamoebae containing 0.308 ± 0.002 mg glycogen/10^8 cells, yet there are no significant differences, either qualitative or quantitative, in the accumulation and decay of either trehalase or T6P synthetase activities during the development of such cells. The rate and extent of trehalose synthesis during the development of axenically-grown myxamoebae is therefore not controlled by the

cellular content of these enzymes. However, the cellular pool sizes of UDPG and G6P are approximately three times greater in myxamoebae initially containing 6.58 ± 0.46 mg glycogen/10^8 cells than in myxamoebae initially containing 0.24 ± 0.03 mg glycogen/10^8 cells, suggesting that the rate of trehalose synthesis may be controlled via substrate availability. Indeed, the increases in substrate concentrations observed are in the correct range to influence markedly the rate of trehalose synthesis (see RESULTS).

In summary, the data presented in this chapter indicate that the rate and extent of trehalose synthesis during the development of axenically-grown cells are controlled not by the cellular content of T6P synthetase (which is not working at maximum velocity)nor trehalase, but more likely by the cellular pool sizes of UDPG and G6P, the substrates for T6P synthetase. This obviously does not rule out control via allosteric effectors although at present none is known for this pathway.

It is interesting that the biphasic nature of trehalose synthesis is maintained even when the total amount of trehalose synthesised varies by a factor of four. This raises the question of what controls the initiation of each burst of trehalose synthesis and what causes the cessation of net synthesis from aggregation until culmination. The accumulation of trehalose and the relevant enzymes and substrates during the development of axenically-grown myxamoebae are shown schematically in Fig. 28. The first burst of trehalose synthesis could be initiated by the decline in intracellular trehalase activity which occurs at this time, but in fact it is

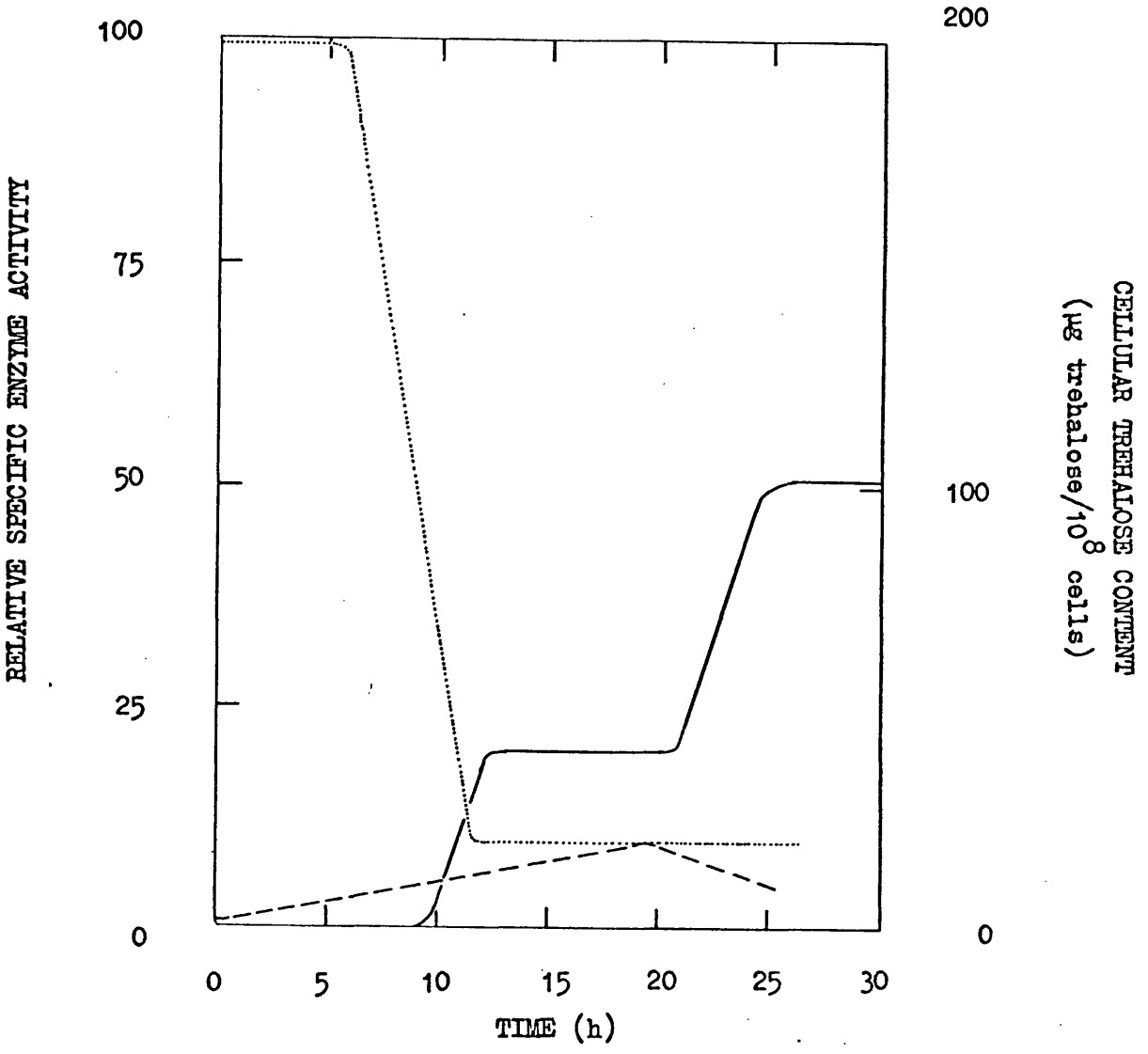


FIG.28 Schematic Representation of The Changes in Trehalose Content, Trehalase Specific Activity, and Trehalose-6-Phosphate Specific Activity during The Development of Axenically-grown Myxamoebae.

..... : trehalase activity

- - - - - : trehalose-6-phosphate synthase activity

——— : cellular trehalose content

doubtful whether this trehalase is able to attack trehalose *in vivo*, or at least *in vivo* activities must be much lower than those observed *in vitro*, since at all times during the development of axenically-grown myxamoebae, trehalase is equal to or in excess of T6P synthetase activity and so one should not observe any accumulation of trehalose. This point will be discussed in more detail later.

Another possibility is that the first burst of trehalose synthesis is initiated by the increase in T6P synthetase activity but in fact, although the specific activity of T6P synthetase is low in vegetative myxamoebae, the total T6P synthetase activity of these cells is almost one quarter the peak total activity attained during development (Fig. 29) making this possibility unlikely. Finally, initiation of trehalose synthesis could be caused by rising UDPG and G6P levels. Certainly, these are much higher during development than during growth (Weeks & Ashworth, 1971) but if this really does initiate trehalose synthesis why does trehalose synthesis cease after aggregation? Obviously, the reasons for the qualitative pattern of trehalose synthesis during the development of axenically-grown myxamoebae is not at all well understood.

During the course of this investigation, Wright and her co-workers came to similar conclusions using bacterially-grown myxamoebae. There are several important differences in developmental trehalose accumulation between bacterially-grown cells strain NC-4 and axenically-grown cells strain Ax-2:

(1) Bacterially-grown cells may possess low levels of trehalose (Ceccarini & Filosa, 1965);

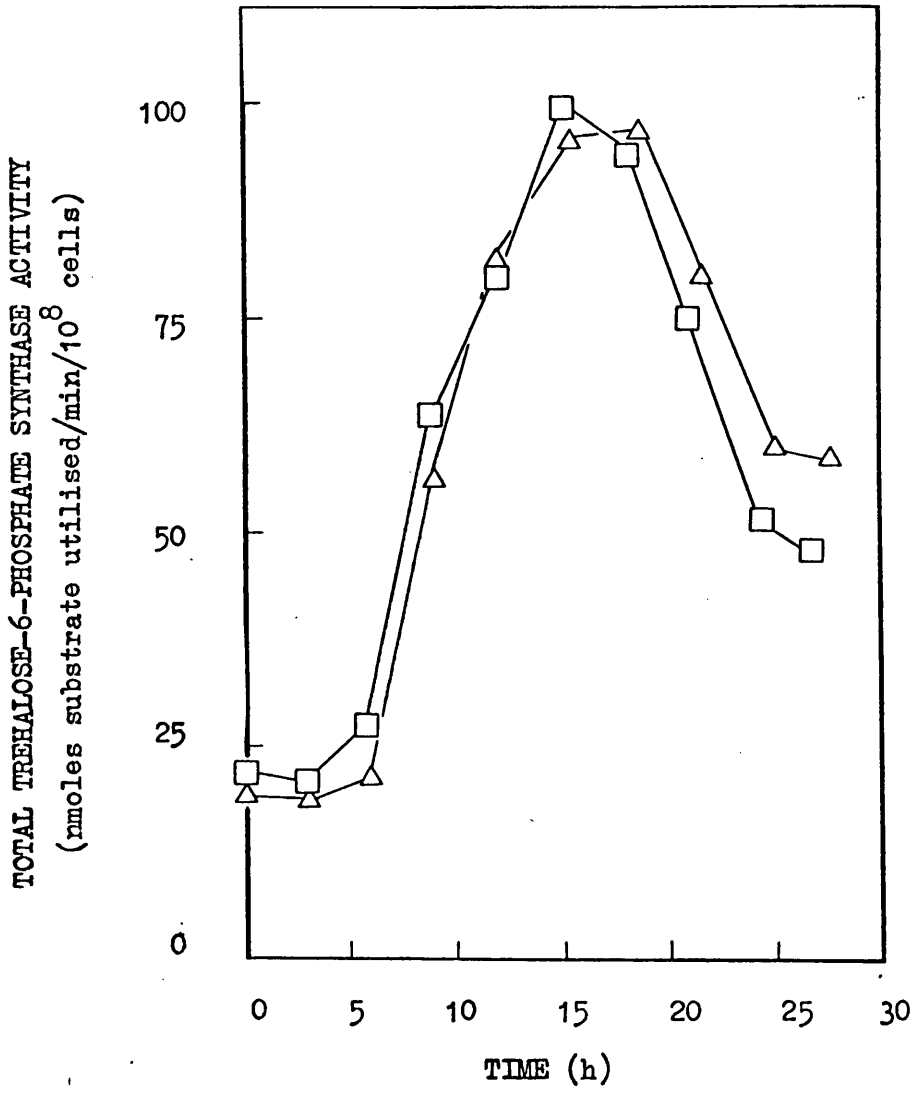


FIG.29 Variation in Trehalose-6-Phosphate Synthase Activity during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained o.29mg glycogen/10^8 cells

△ ; myxamoebae initially contained 5.95mg glycogen/10^8 cells

axenically-grown cells lack trehalose completely.

(2) At all stages of development, bacterially-grown cells possess trehalase activities less than one tenth those of axenically-grown cells (Ceccarini, 1967; this chapter).

(3) Bacterially-grown cells accumulate trehalose only during fruiting body construction (Ceccarini & Filosa, 1965); axenically-grown cells accumulate trehalose during late aggregation as well as during fruiting body construction.

Whether these differences are due to strain differences between Ax-2 and NC-4, a result of different growth conditions, or some other reason is not known. Figure 30 is a schematic representation of changes in the important components of the trehalose synthetic and degradative pathways during the development of bacterially-grown myxamoebae.

Using this data, Wright & Marshall (1971) expanded the computer model of Wright et al., (1968b) to include the synthesis and breakdown of trehalose (Fig. 31). An IBM 360/65 computer was programmed according to this expanded model using in vivo determined metabolite concentrations and in vitro determined enzymic kinetic parameters, and used to explore the effects of changing trehalase and T6P synthetase activities.

One odd assumption of the model was that some of the trehalose present in bacterially-grown myxamoebae (Ceccarini & Filosa, 1965) is degraded early on in development. In fact, only in germinating myxamoebae is the intracellular trehalose level known to fall markedly (Ceccarini, 1967); in amoebae which have been growing

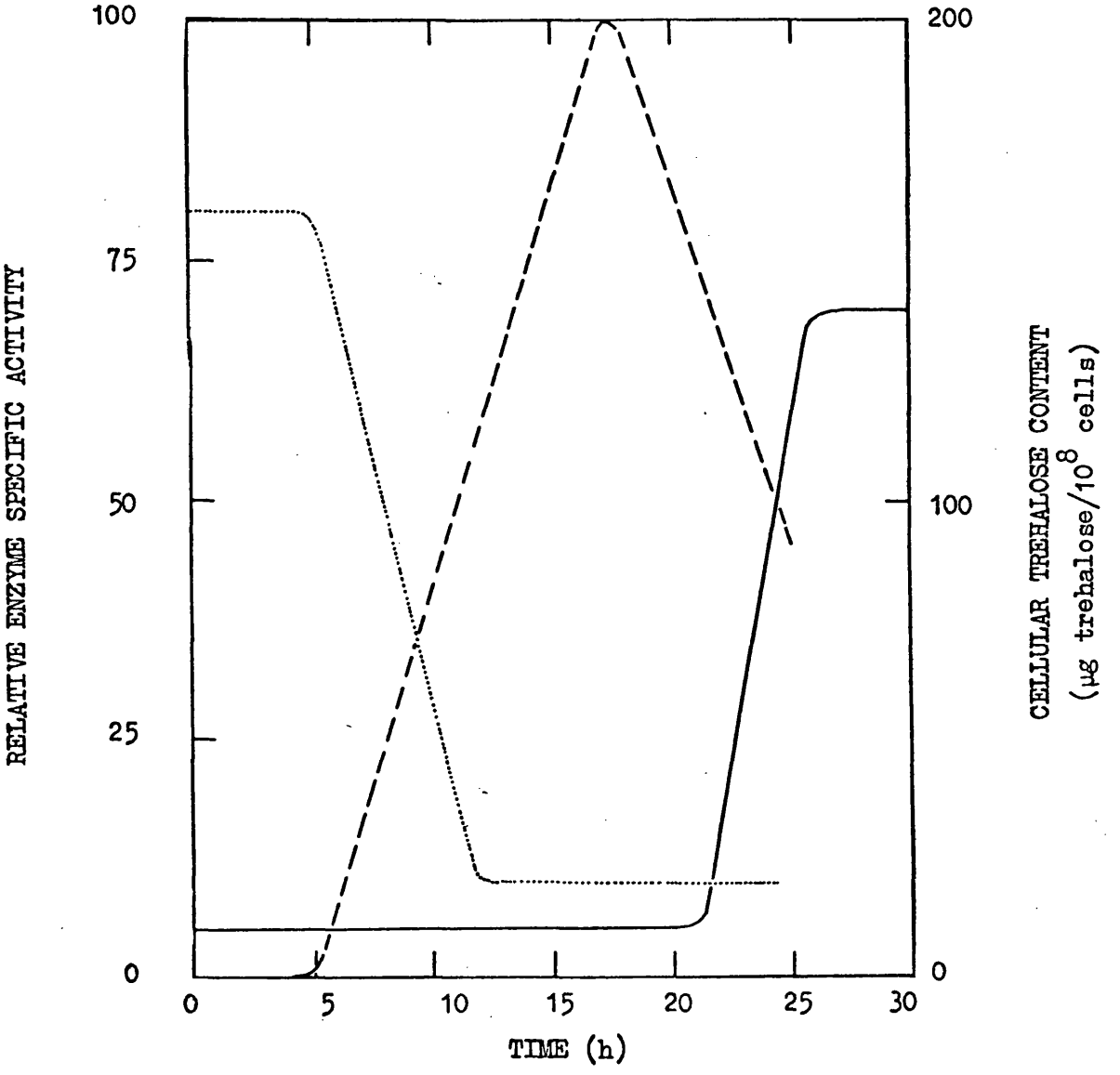


FIG. 30 Schematic Representation of The Changes in Trehalose Content, Trehalase Specific Activity, and Trehalose-6-Phosphate Synthase Specific Activity during The Development of Bacterially-grown Myxamoebae.

····· ; trehalase activity (from Ceccarini 1967)

– – – – ; trehalose-6-phosphate synthase activity (from Roth @ Sussman 1968)

——— ; cellular trehalose content (from Ceccarini @ Filosa 1965, Sussman @ Sussman 1969)

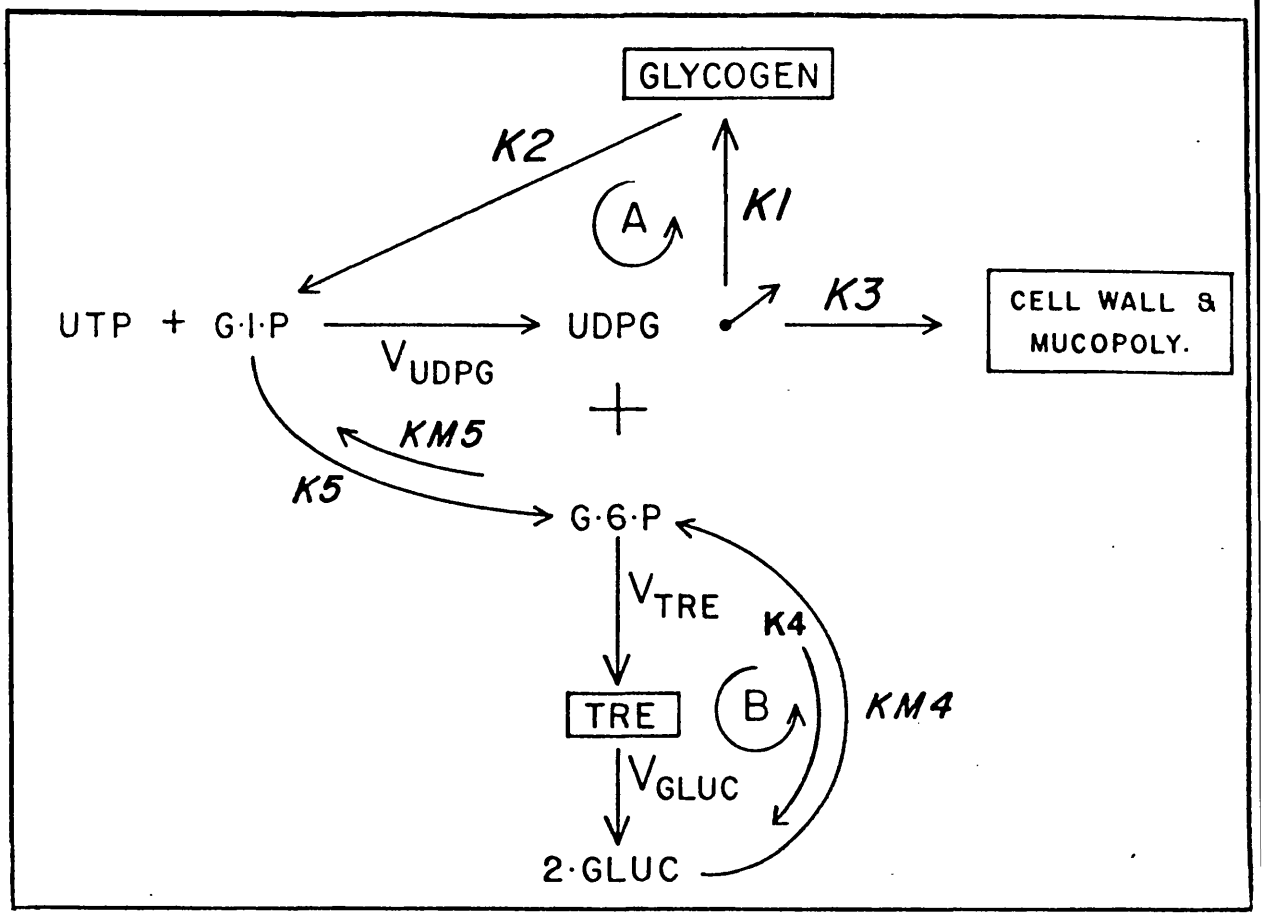


FIG. 31 The Kinetic Model as expanded to simulate Trehalose Synthesis (Wright & Marshall 1972).

V_{UDPG} : the rate of UDPG synthesis (involving UDPG pyrophosphorylase)

V_{TRE} : the rate of trehalose synthesis (involving T6P synthase and T6P phosphatase)

V_{GLUC} : the rate of trehalose degradation (involving trehalase)

K1 : the rate constant for glycogen synthesis

K2 : the rate constant for glycogen degradation

K3 : the rate constant for mucopolysaccharide and cell wall polysaccharide synthesis

K4 : the rate constant for a G6P phosphatase reaction

KM4 : the rate constant for a glucokinase reaction

K5, KM5 : the rate constants for the phosphoglucomutase reaction

Cycle A is concerned with glycogen synthesis and degradation

Cycle B is concerned with trehalose synthesis and degradation

on bacteria for several generations the trehalose level is low and remains approximately constant throughout development until trehalose synthesis occurs during fruiting body construction (Ceccarini & Filosa, 1965).

Only two models were found to account for the *in vivo* changes in metabolite concentrations and end products (including the assumed degradation of trehalose early on in development).

The first ("turnover") model assumed that trehalose synthetic activity remains constant during development but that trehalase decreases in the manner reported on the basis of *in vitro* assays (Ceccarini, 1967). According to this model, trehalose turns over prior to fruiting body construction.

The second ("no turnover") model assumed a very low rate of trehalose degradation (approx. 1% of the trehalase activity observed *in vivo*) to account for the degradation of myxamoebal trehalose early on in development and maintained this low rate throughout development. Under these conditions, in order to observe the changes in metabolite concentrations and end-products reported to occur *in vivo*, the model predicted that trehalose synthetic activity is absent prior to fruiting body construction and then, over a period of 1 h, increases 100-fold. Sargent & Wright (1971) then measured the *in vivo* rate of trehalose synthesis during the development of bacterially-grown myxamoebae and found that turnover of trehalose prior to fruiting body construction is insignificant. This ruled out the first successful computer model which incorporated trehalose turnover prior to sorocarp

formation. In agreement with the second ("no turnover") model, the rate of trehalose synthesis is low until fruiting body construction and then increase approximately 100-fold over an approximately 2 h period (Sargent & Wright, 1971).

Wright and her co-workers therefore believe that the changes in trehalose synthetic and degradative activities reported on the basis of in vitro assays (Ceccarini, 1967; Roth & Sussman, 1968) do not occur in vivo during the development of bacterially-grown myxamoebae. Thus, although trehalase decreases greater than ten-fold in activity during aggregation (Ceccarini, 1967), the successful computer model predicts a low (1% of the in vitro activity) rate of trehalose degradation present at the same level throughout development. Similarly, the computer model and in vivo measurements suggest that trehalose synthesis does not occur until fruiting body construction whereas T6P synthetase activity is reported to increase during slug migration and actually begins to decrease before sorocarp formation (Roth & Sussman, 1968). Moreover, the maximum rate of trehalose synthesis predicted by the model is 100-fold higher than the T6P synthetase activity present at this time (Roth & Sussman, 1968).

The general conclusion of Wright's analysis is that the flux through the trehalose synthetic pathway is not controlled by the cellular content of T6P synthetase or of trehalase (as measured by in vitro assays); in complete agreement with the results described in this chapter using axenically-grown myxamoebae.

However, there are two criticisms of the successful computer model of Wright & Marshall (1971).

Firstly, the model incorporates the data that trehalose is present in vegetative myxamoebae grown on bacteria (Ceccarini & Filosa, 1965) and is degraded during the early stages of development. Now, Ceccarini & Filosa (1965) obtained their myxamoebae by inoculation of agar plates with *E. coli* B/r/ slime mould spore suspensions and then harvesting and washing the myxamoebae after 36-40 h growth. So, it is possible that the low level of trehalose observed in the cells was due to contamination with ungerminated spores. In support of this, in only one of the two studies made by these authors was trehalose observed to be present in bacterially-grown myxamoebae. Moreover, in the study in which trehalose was reported to be present in vegetative myxamoebae, there was little if any net degradation of trehalose during development. Thus there appears to be no need to include trehalose degradative capacity in the model at all.

Secondly, the model assumes the absence of gluconeogenesis but I have already reviewed the evidence for believing that gluconeogenesis is an important phenomenon during the development of axenically-grown, and probably bacterially-grown, myxamoebae (Chapter II).

Both the results of Sargent & Wright (1971) and the data presented in this chapter suggest that the high cellular trehalase activity detected using *in vitro* enzyme assays cannot be present *in vivo*. Either trehalase is modified *in vivo* so that it is much less active, or it is unable to attack trehalose synthesised by the developing cells.

It is notable that the pH optimum of purified *D. discoideum* trehalase is 5.6 (Ceccarini, 1966) and this is the pH of *in vitro* trehalase

assays, but the enzyme is known to be soluble (Weeks & Ashworth, 1972) and, since the pH of the cytoplasm is presumably nearer neutrality, this will reduce the activity of the enzyme, but not sufficiently to prevent trehalase activity being in excess during development.

A more likely possibility is that trehalose synthesis occurs in some compartment isolated from trehalase action. Now trehalose synthesis is confined to spore cells, so possibly during development, trehalase is lost completely from pre-spore cells but pre-stalk cells retain a low level of activity. The experiments described above, involved in measuring cellular trehalase activity, used entire populations of developing cells without fractionation into pre-spore and pre-stalk cells and therefore would not detect compartmentalisation of this kind, but, in fact, mature non-germinating spores do contain trehalase and at levels comparable to that found in the cells of the migrating slug analysed as a whole (Ceccarini, 1967).

The alternative is intracellular compartmentation, that is, although spores contain both trehalose and trehalase, these may be present in completely separate cellular locations so that trehalase is never able to attack the trehalose. As stated above, trehalase is a soluble enzyme, but there is evidence that trehalose synthesis may occur in specific vesicular compartments isolated from the general cell cytoplasm. Thus, Gregg & Badman (1970) report that spores of D. discoideum, but not stalk cells, possess specific vesicles called spore vesicles which appear after aggregation, are retained in the mature spore, and disappear upon spore germination.

In respect to their specificity to spores and not stalk cells, and the time of their appearance and disappearance, spore vesicles thus correlate well with the presence of trehalose. However, these vesicles have not yet been isolated and examined for trehalose content.

Finally, the biphasic pattern of trehalose synthesis during the development of axenically-grown myxamoebae may be of importance to the problem of determination. As pointed out in the general introduction, development can be subdivided into a phase of determination (during which the fate of the cells is decided) followed by a phase of differentiation (during which the cells undergo biochemical changes resulting in the differentiated state). For example, the development of D. discoideum can be divided into a phase when the cells decide whether to become spore or stalk cells, followed by a phase in which they actually undergo the necessary changes to become spores or stalk cells. However, it is not known with any certainty exactly when during development in D. discoideum the cells have determined their fate. Now, as previously described, trehalose is believed to be found only in spore cells (Ceccarini & Filosa, 1965; Table 18) and therefore appears to be a marker for this cell type. However, during the development of axenically-grown myxamoebae, trehalose is synthesised during aggregation. Therefore, either trehalose is synthesised by both pre-spore and pre-stalk cells during the development of axenically-grown myxamoebae (and possibly later lost from stalk cells during the autolysis which occurs during sorocarp construction (George et al., 1972) or trehalose synthesis is restricted to pre-spore cells, in which case determination must

TABLE 18 The distribution of trehalose in fruiting bodies derived from the development of axenically-grown myxamoebae

Sample	Cellular trehalose content ($\mu g/10^8$ myxamoebae)
Stalk cells	4.63 ± 0.25 (2)
Spores	321.82 ± 34.24 (2)

Results are given as means $\pm$ S.E.M.

Figures in brackets refer to the number of determinations

Comparison of the trehalose content of stalk cells and spores on the basis of cell number is not feasible since the stalk cells are encased in the stalk matrix rendering them impossible to count. Likewise, protein content has not been used as a basis because the stalk cells are known to metabolise more of their protein during development than spore cells (Gregg et al., 1954). Thus, comparison has been made on the basis of myxamoebal number; for example, the stalk cell trehalose content of 4.63 ± 0.25 (2) $\mu g/10^8$ myxamoebae should be interpreted as the trehalose content of all the stalk cells produced by the development of 10^8 myxamoebae.

occur at or before aggregation. Experiments with mutant marker cells (Bonner, 1959), spore protein specific antibodies, and tritiated thymidine labelled cells (Takeuchi, 1969) and studies of the density (Takeuchi, 1969; Bonner et al., 1971) and structure (e.g. Gregg & Badman, 1971) of developing cells of D. discoideum are all in agreement with the latter suggestion. It is clear that future studies on the site of trehalose synthesis during the development of axenically-grown cells could have important implications for both a biochemical and cell biological understanding of development.

In conclusion, our understanding of the controls operating on any one end-product synthetic pathway is fragmentary, but at least for developmental trehalose accumulation it is clear that the cellular contents of key synthetic and degradative enzymes are not the controlling factors.

SUMMARY

1. The relationship between myxamoebal glycogen content and the amount of end-product saccharides synthesised during the development of axenically-grown myxamoebae was studied with the intention of clarifying the control of end-product saccharide synthesis.

2. Evidence was obtained which suggested that the syntheses of end-product saccharides are not coordinately controlled but rather that each pathway is subject to individual control.

3. The accumulation of trehalose, chosen for further study for various reasons, was found to be controlled not by the cellular enzyme content of key synthetic and degradative enzymes but more likely by the cellular concentrations of UDPG and G6P, the substrates for trehalose synthesis.

4. These results were discussed in the light of our present knowledge concerning the control of end-product saccharide synthesis during the development of D. discoideum.

CHAPTER IV

THE INTERRELATIONSHIP OF GLYCOGEN, RIBONUCLEIC ACID, AND PROTEIN METABOLISM DURING DEVELOPMENT

CHAPTER IV

THE INTERRELATIONSHIP OF GLYCOGEN, RIBONUCLEIC ACID AND PROTEIN METABOLISM DURING DEVELOPMENT

INTRODUCTION

One characteristic common to most, perhaps all, developing systems is that they are essentially isolated from the external environment by cellular permeability barriers allowing the complex, precise events of morphogenesis to occur in an easily regulated intracellular environment, unaffected by unpredictable changes in the extracellular milieu. However, the price paid for this independency is that the energy and precursor requirements of development must be satisfied from endogenous sources and this necessitates that endogenous material be extensively degraded. Protein and RNA are the compounds most usually utilised for this purpose (Heatley & Lindahl, 1937; Foster & Perry, 1954; Backström, 1959; Chet & Rusch, 1969) although carbohydrates may also be mobilised (Heatley & Lindahl, 1937; Zielinski, 1939; Cantino & Goldstein, 1961; Karlson & Sekeris, 1964).

The cellular slime mould is no exception to this general pattern. Studies of the development of bacterially-grown myxamoebae of D. discoideum have shown that protein and RNA are rapidly degraded during this process (e.g. Gregg et al., 1954; White & Sussman, 1961; Hanks, 1967).

Data presented in Chapters I and II indicate that myxamoebae grown in axenic medium containing added glucose will accumulate large amounts of glycogen, and that during development this is metabolised to

carbon dioxide, presumably yielding metabolically useful energy. If the main purpose of protein and RNA breakdown during development is normally to supply the energy for development (as suggested by the work of Gregg et al., 1954; Wright, 1963; Hanks, 1967) one might expect that the use of glycogen to this end will spare such breakdown or at least delay it until exhaustion of the cellular carbohydrate reserve. [as during starvation in other microbial systems (Dawes & Ribbons, 1964; Mizunuma, 1963)]. Alternatively, protein and RNA degradation may play a role in development as well as to provide a source of energy, in which case the availability of another cellular source of energy may have no effect on this breakdown. This chapter, by seeking to solve the above problem, probes the interrelationship between the metabolism of protein, RNA and glycogen during development.

MATERIALS

Bovine serum albumin, ribose, glycine and ninhydrin were obtained from Sigma (London) Ltd., London W.5, U.K.; glutamate dehydrogenase was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K.; and orcinol was obtained from Fison's Scientific Apparatus Ltd., Loughborough, Leics., U.K. All other chemicals were of the highest purity commercially available and obtained either from B.D.H. Chemicals Ltd., Poole, Dorset, U.K., or Fison's Scientific Apparatus Ltd., Loughborough, Leics., U.K., or from sources quoted in the previous chapters.

METHODS

Cell Fractionation and Determination of DNA, RNA and Protein

At various times during the developmental phase, cells were harvested into ice cold water to give a final suspension of 2.5×10^8 cells in 4 ml water. These samples were stored at -35°C. After being thawed, samples were made up to 5 ml with water and sonicated with a 100 W MSE ultrasonic disintegrator (peak to peak amplitude 9 μm) for either four 15 second periods (samples 0-16 h) or for twelve 15 second periods (samples 16-30 h), with continuous cooling in an ice salt bath to maintain samples at a low temperature. A sample of this extract was assayed for total cellular protein by the method of Lowry. Cold 50% ($^w/_v$) trichloroacetic acid (TCA) (1 ml) was added to another 4 ml of extract and the mixture incubated for 1 h at 0°C. The resulting precipitate was centrifuged (1000 g, 20 min.). The pellet was washed with 2 ml of cold 10% ($^w/_v$) TCA and the supernatants from the two centrifugations pooled and used for estimation of cold TCA-soluble protein by the method of Lowry et al. (1951). The pellet was resuspended in 2.5 ml of 5% ($^w/_v$) TCA and heated at 90°C for 30 min to hydrolyse the DNA and RNA. The hydrolysate was centrifuged (1000 g, 20 min) and the supernatant (hot TCA-soluble fraction) assayed for DNA (by the method of Giles and Meyer (1965) as described in Chapter II), RNA by the orcinol reaction (Mejbaum, 1939) and protein (by the method of Lowry et al., 1951). The TCA-insoluble pellet was dissolved in 4 ml N NaOH and assayed for protein by the method of Lowry et al.(1951).

The standard solutions for DNA, RNA and protein estimations were prepared from deoxyribose, ribose and anhydrous bovine serum albumin(BSA) respectively.

Determination of Cellular Amino Acid

At various times during the developmental phase, cells were harvested into ice cold water to give approximately 2.0×10^8 cells per 4 ml water. These samples were stored at $-35^{\circ}C$. Cell extracts were prepared by sonication as above and amino acids were extracted from 4 ml of the sample by boiling for 30 min. Following incubation at $0^{\circ}C$ for 15 min., insoluble cell debris was removed by centrifugation (1000g, 10 min.) and the supernatant analysed for amino acid by the ninhydrin method (Mitchell & Moyle, 1953). Ammonia in the samples was responsible for some of the ninhydrin reaction and was corrected for by assay of ammonia by the method of Levitzki and the subtraction of the ninhydrin colour given by that amount of ammonia present in the sample, from the total sample ninhydrin colour. The standard solutions were prepared from glycine and ammonium chloride.

Determination of TCA-soluble Pentose Material

TCA-soluble fractions were prepared as described above and assayed for pentose using the orcinol reaction (Mejbaum, 1939).

Determination of Extracellular Protein, Amino Acid, U.V. absorbing material and Ribose

Myxamoebae were allowed to develop as described under

Methods, Chapter I except that the PDF used did not contain streptomycin since this interferes with the assay for protein according to Lowry et al. (1951). At various times during the developmental phase, PDF was harvested from 8 millipore filter supports by squeezing the supporting pads using protective gloves. The collected PDF was centrifuged (1000g, 10 min) to remove cellulose fibres and then stored at -35°C. After thawing, the protein content of the PDF was determined by the method of Lowry et al. (1951) with anhydrous BSA as standard, and the amino acid content determined and corrected for the presence of ammonia as described under "Determination of Cellular Amino Acid". Other samples of the PDF were used to determine the absorbance at 260 nm and 280 nm and to assay PDF ribose content by the orcinol reaction (Mejbaum, 1939) using ribose as standard. For all these assays, correction was made for the reaction or absorbance given by the components of PDF itself.

Determination of Ammonia

Myxamoebae were allowed to differentiate on 27mm diameter Millipore filter supports in closed Conway dishes as described in Chapter II (p. 19) except that no NaOH was present in the outer walls of the Conway dishes. At intervals during the developmental phase, PDF was collected by squeezing the Millipore membrane supporting,pads, centrifuged (1000g, 10 min.) to remove cellulose fibres, and stored at -35°C. After slow thawing, the PDF was assayed for ammonia using glutamic dehydrogenase as described by Levitzki (1971) correcting for

the initial ammonia content of the PDF.

Growth and Development of ^{14}C-Aspartate-labelled Myxamoebae

^{14}C-aspartate-labelled myxamoebae were grown and allowed to develop as described under METHODS in Chapter II.

Radioactivity of PDF

The PDF used for the development of ^{14}C-aspartate-labelled cells was collected at intervals during the developmental phase as described above. After removal of the cellulose fibres by centrifugation, samples were added to 10 ml of a Triton X-100 - toluene mixture (Fox, 1968) and counted in a Packard Tricarb Scintillation spectrometer, as described in Chapter II.

Determination of Cellular Protein Specific Radioactivity

Protein specific radioactivity was determined as described under Methods in Chapter II.

Determination of Carbon Dioxide Radioactivity

Carbon dioxide radioactivity was determined as described under Methods in Chapter II.

RESULTS

In order to correct for variable cell recoveries from Millipore filter supports during development, the values for cellular protein and RNA contents were corrected to mg protein per 10^8 cells and mg RNA (in ribose equivalents) per 10^8 cells respectively, by measuring, in the same sample, the amount of DNA and assuming that:-

(1) the DNA content of 10^8 myxamoebae is 17.86 $\mu g/10^8$ cells

and (2) the DNA content per cell does not change during development.

Evidence for believing both these assumptions are valid has been presented in Chapter II.

Protein Metabolism During Development

During the development of axenically-grown myxamoebae containing less than about 0.3 mg glycogen per 10^8 cells, the amino acid pool and TCA-soluble protein pool are rapidly metabolised and soon reach low levels which are maintained throughout the rest of the developmental phase (Figs. 32, 33 □). TCA-insoluble protein is also degraded (Fig. 34 □) but at a slower rate than TCA-soluble protein. The data for the decay of TCA-insoluble protein (Fig. 34) and total cellular protein* (Fig. 35) were analysed statistically by regression analysis and found to fit the pattern of decay indicated rather than an exponential decay. Thus, in Table 20, rates of protein degradation refer to the linear rate of total cellular protein (cold TCA-soluble plus TCA-insoluble protein) decay observed during the 4-22 h (approx.) period of development.

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* See footnote on p.105

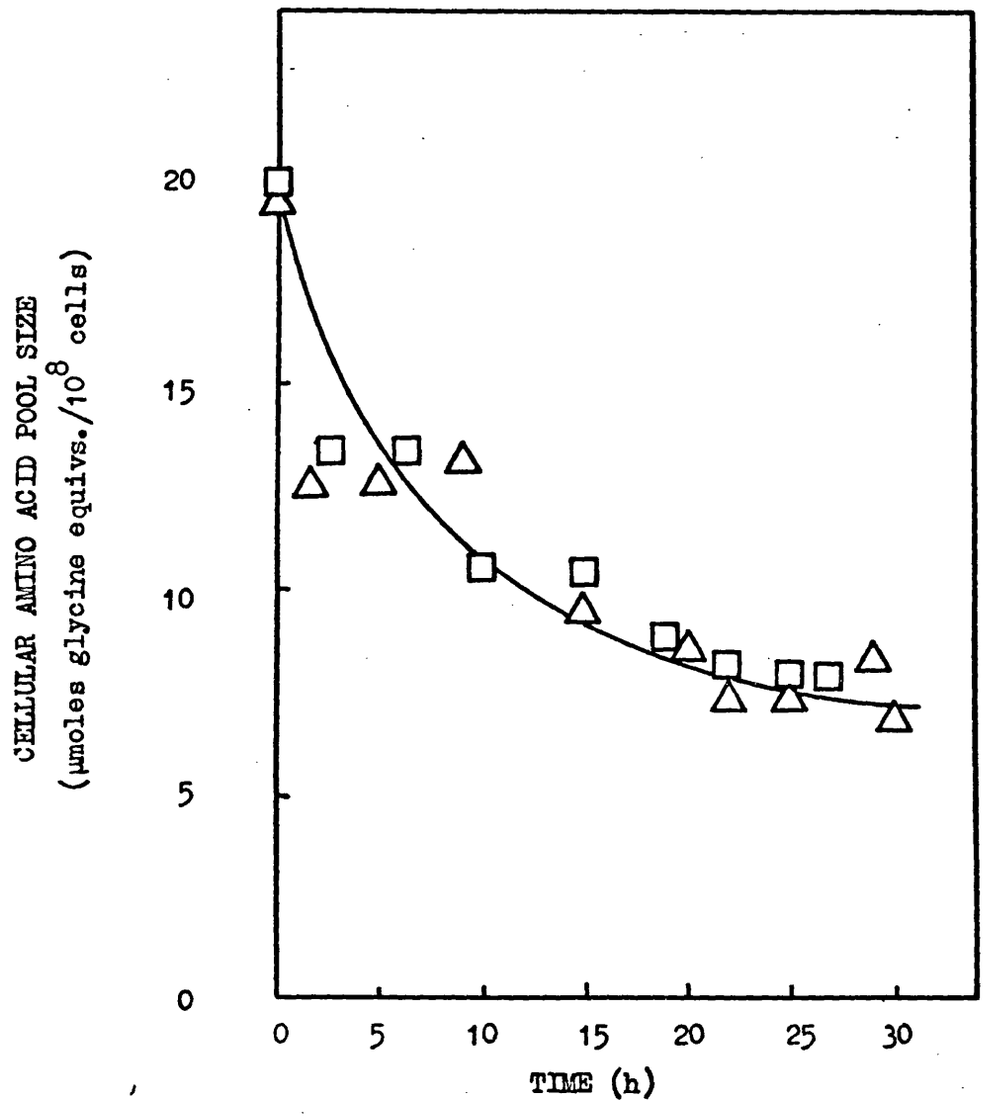


FIG. 32 Changes in The Cellular Amino Acid Pool Size during The Development of Axenically-grown Myxamoebae.

□; myxamoebae initially contained 0.094mg glycogen/10^8 cells

△; myxamoebae initially contained 2.660mg glycogen/10^8 cells

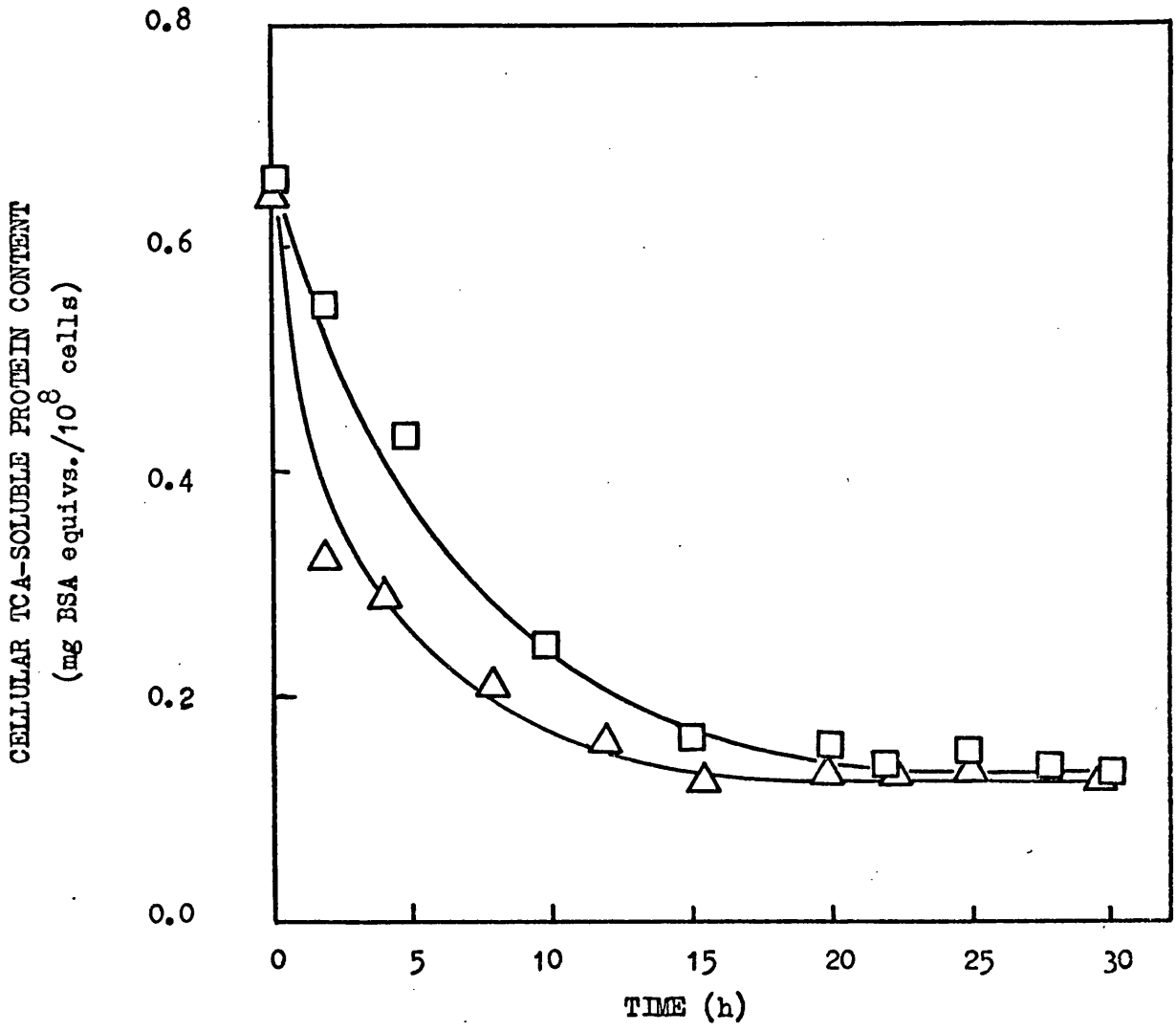


FIG.33 Changes in The Cellular TCA-soluble Protein Content during The Development of Axenically-grown Myxamoebae.

□; myxamoebae initially contained 0.046mg glycogen/10^8 cells

△; myxamoebae initially contained 5.560mg glycogen/10^8 cells

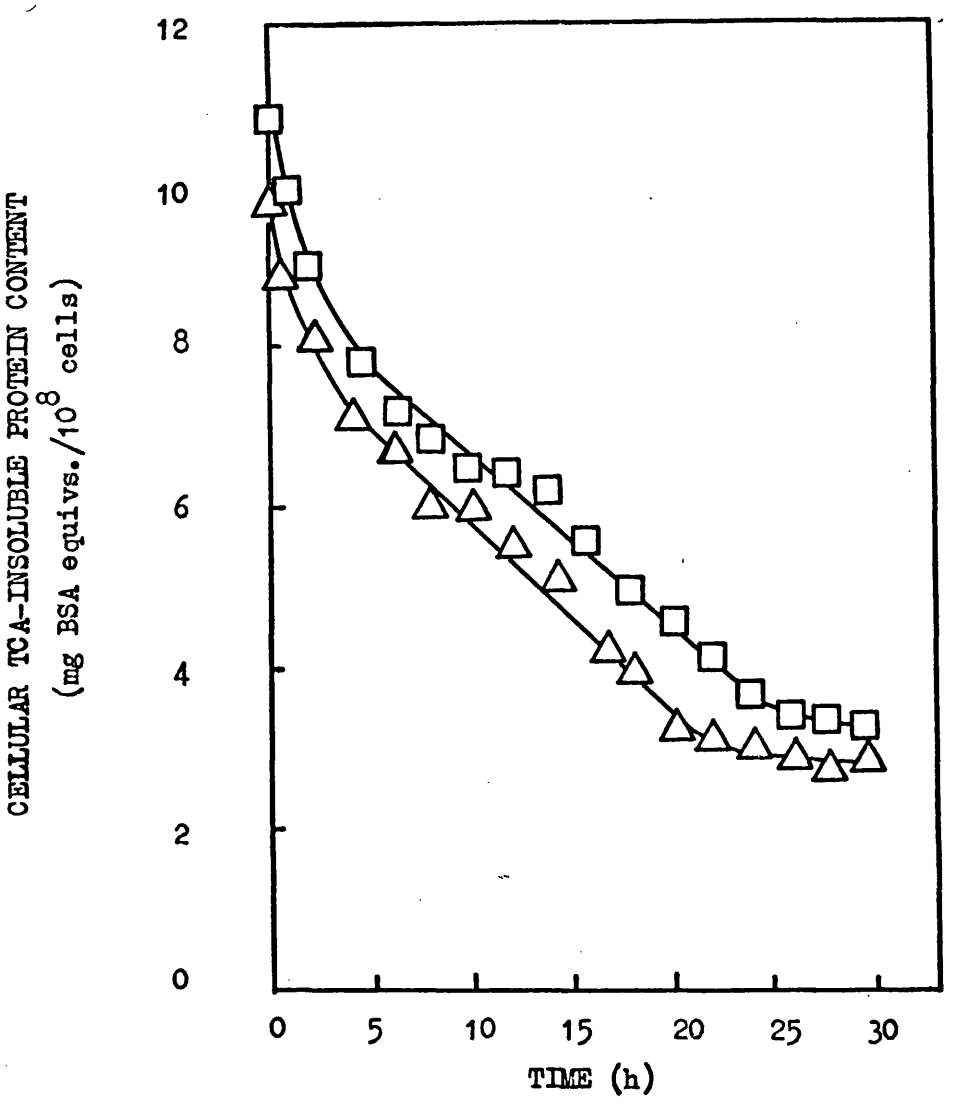


FIG. 34 Charges in The Cellular TCA-insoluble Protein Content during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.32mg glycogen/10^8 cells

△ ; myxamoebae initially contained 1.24mg glycogen/10^8 cells

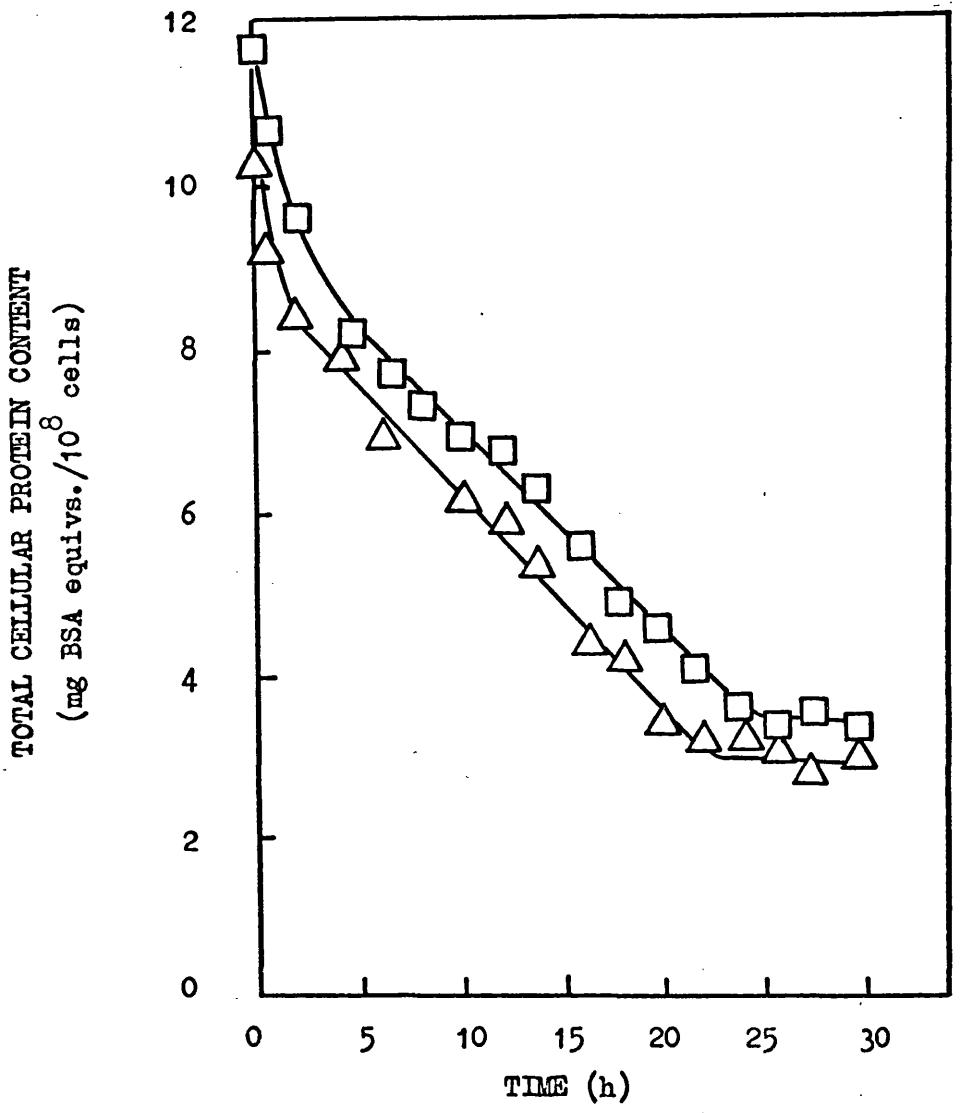


FIG. 35 Changes in The Total Cellular Protein Content during The Development of Axenically-grown Myxamoebae.

□ ; myxamobae initially contained 0.32mg glycogen/10^8 cells

△ ; myxamoebae initially contained 1.24mg glycogen/10^8 cells

TABLE 19 The cellular content of hot TCA soluble protein during the development of axenically-grown myxamoebae

Developmental time (h)	$\frac{\text{Hot TCA soluble protein}}{\text{Total cellular protein}} \times 100\%$	
	*NS	*G
0	3.89	6.27
1	3.93	6.07
2	3.82	5.73
5	3.77	6.03
10	3.78	6.47
15	3.76	5.78
20	4.83	6.33
25	4.40	6.95
30	5.41	7.59

*NS: myxamoebae initially contained 0.094 mg glycogen/10^8 cells

*G : myxamoebae initially contained 2.227 mg glycogen/10^8 cells

TABLE 20: The relationship between myxamoebal glycogen content and the rate and extent of protein degradation during the developmental phase.

Myxamoebal Glycogen content ($mg/10^8$ cells)	Myxamoebal protein content ($mg/10^8$ cells)	Sorocarp (26h) protein content ($mg/10^8$ cells)	Amount of protein degraded during the 0-26h period of development ($mg/10^8$ cells)	Rate of protein degradation ($mg/10^8$ cells/h.)	Percentage of myxamoebal protein degraded during the 0-26h period of development ($mg/10^8$ cells)	*Percentage Rate	*Percentage Amount
0.071±0.014 (3)	12.26±0.45 (3)	3.67±0.32 (3)	8.43±0.26 (3)	0.256±0.018 (3)	67.83	100.00	100.00
0.321±0.000 (2)	12.45±0.71 (2)	3.82±0.24 (2)	8.64±0.46 (2)	0.238±0.004 (2)	69.55	92.97	102.49
1.176±0.064 (2)	10.35±0.10 (2)	3.29±0.21 (2)	7.07±0.12 (2)	0.235±0.050 (2)	71.65	91.80	83.87
1.736±0.244 (2)	10.79±0.36 (2)	4.28±0.22 (2)	6.48±0.12 (2)	0.227±0.032 (2)	63.35	88.67	76.87
2.670 (1)	11.78 (1)	4.95 (1)	6.83 (1)	0.216 (1)	60.20	84.38	81.02
3.620 (1)	10.77 (1)	4.07 (1)	7.91 (1)	0.253 (1)	73.70	98.83	93.83
5.560 (1)	11.16 (1)	4.25 (1)	6.91 (1)	0.208 (1)	62.00	81.25	81.97

Results are given as means ± S.E.M.

Figures in brackets refer to the number of experiments.

Each rate was calculated using 10 experimental points, on average; 6-14

*Percentage Rate = $\frac{\text{Rate of protein degradation by myxamoebae during development}}{\text{Rate of protein degradation by myxamoebae initially containing } 0.071 \pm 0.014 \text{ (3) mg glycogen}/10^8 \text{ cells.}} \times 100\%$

*Percentage Amount = $\frac{\text{Amount of protein degraded by myxamoebae during the 0-26h. period of development}}{\text{Amount of protein degraded by myxamoebae initially containing } 0.071 \pm 0.014 \text{ (3) mg glycogen}/10^8 \text{ cells during the 0-26h. period of development.}} \times 100\%$

When axenically-grown myxamoebae contain increased levels of glycogen, protein is still degraded to approximately the same extent and at approximately the same rate as observed during the development of myxamoebae initially containing less than 0.3 mg glycogen per 10^8 cells (Figs. 33,34,35Δ, Table 20). There is also a similar utilisation of intracellular amino acids (Fig. 31Δ).

Extracellular Protein

During the first few hours of development, total cellular protein decreases rapidly (Fig. 35) and only later assumes a steady linear rate of decay.

In order to determine whether this initial rapid loss of cellular protein is due to intracellular protein utilisation or to protein excretion, the protein present in the extracellular fluid was

---

* Throughout the developmental phase, the Folin positive material present in the hot TCA-soluble fraction remained a constant percentage of the total cellular protein (Table 19). Since the hot TCA-soluble fraction contains hydrolysed cellular DNA and RNA, and since guanine, xanthine and other compounds usually extracted into this fraction are known to react with the Folin reagent (Lowry et al., 1951), "total cellular protein" in Fig. 35 was taken to be the sum of the cold TCA-soluble and TCA-insoluble protein fractions. Control experiments show that over 90% of cellular protein is found in these two fractions.

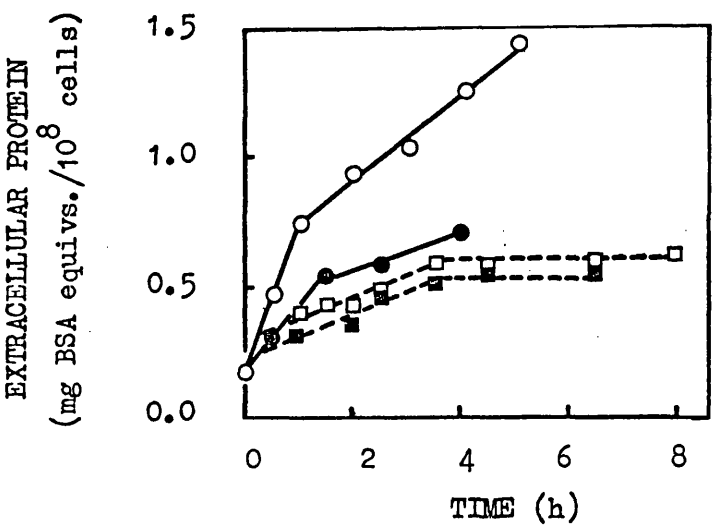
estimated. Myxamoebae were harvested by the standard procedure (Chapter I) and resuspended in water. Some of these cells were allowed to differentiate on Millipore membrane supports whilst others were periodically shaken in water. Intracellular and extracellular Folin-positive material was determined at intervals. The data indicate that myxamoebae extrude Folin-positive compounds upon resuspension in water and that this extrusion continues, although sometimes at a lower rate, for approximately 2-3 h. after the cells are deposited on Millipore membrane supports (Fig. 36) with a concomitant fall in intracellular Folin-positive material (Fig. 37). The absolute amount of material excreted is variable but usually less than 1.0 mg BSA equivalents per 10^8 cells (Fig. 38). During the course of these experiments cells were washed from the Millipore filter supports and counted, showing that less than 5% of cells lysed whilst the cells were on the Millipore filter supports and therefore the protein in the extracellular PDF was almost certainly excreted protein. [Cells suspended in water are stable for at least 4 h. if gently shaken periodically to maintain aerobic conditions but after this time lysis starts to occur.]

Since the majority of excreted Folin-positive material is excreted between harvesting the myxamoebae and 3 h. after deposition of the cells on Millipore membrane supports, it is undoubtedly responsible for a significant portion of the rapid decrease in intracellular protein observed at this time (Fig. 35).

The presence of high levels of myxamoebal glycogen has no effect on the extent of excretion of Folin-positive material (Figs.36,38) or on the initial rapid decrease in cellular protein content (Figs.35,37).

FIG. 36 Excretion of Cellular Protein by Axenically-grown Myxamoebae.

Fig. 36(a)



Two experiments are shown:-

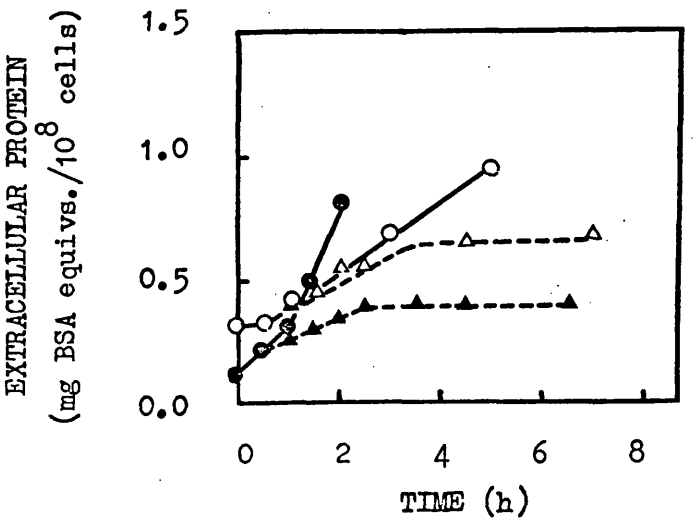
○ ; excretion of protein by myxamoebae initially containing 0.30mg glycogen/10^8 cells and shaken in water

□ ; excretion of protein by myxamoebae initially containing 0.30mg glycogen/10^8 cells and placed on Millipore filters

● ; excretion of protein by myxamoebae initially containing 0.28mg glycogen/10^8 cells and shaken in water

▣ ; excretion of protein by myxamoebae initially containing 0.28mg glycogen/10^8 cells and placed on Millipore filters

Fig. 36(b)



Two experiments are shown:-

○ ; excretion of protein by myxamoebae initially containing 1.53mg glycogen/10^8 cells and shaken in water

△ ; excretion of protein by myxamoebae initially containing 1.53mg glycogen/10^8 cells and placed on Millipore filters

● ; excretion of protein by myxamoebae initially containing 1.95mg glycogen/10^8 cells and shaken in water

▲ ; excretion of protein by myxamoebae initially containing 1.95mg glycogen/10^8 cells and placed on Millipore filters

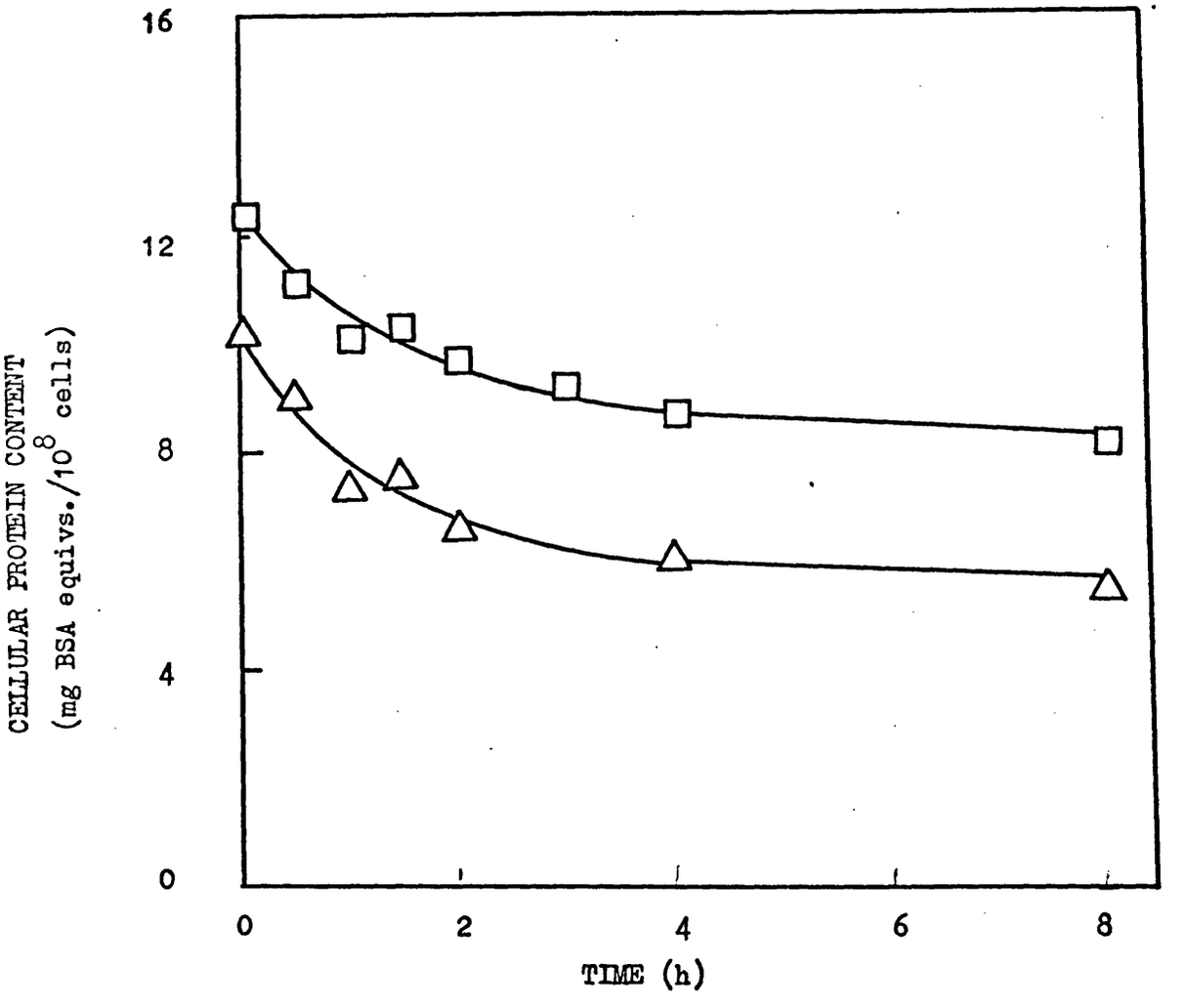


FIG.37 Changes in Cellular Protein Content during The Early Hours of Development of Axenically-grown myxamoebae.

□ ; myxamoebae initially contained 0.28mg glycogen/10^8 cells

△ ; myxamoebae initially contained 1.95mg glycogen/10^8 cells

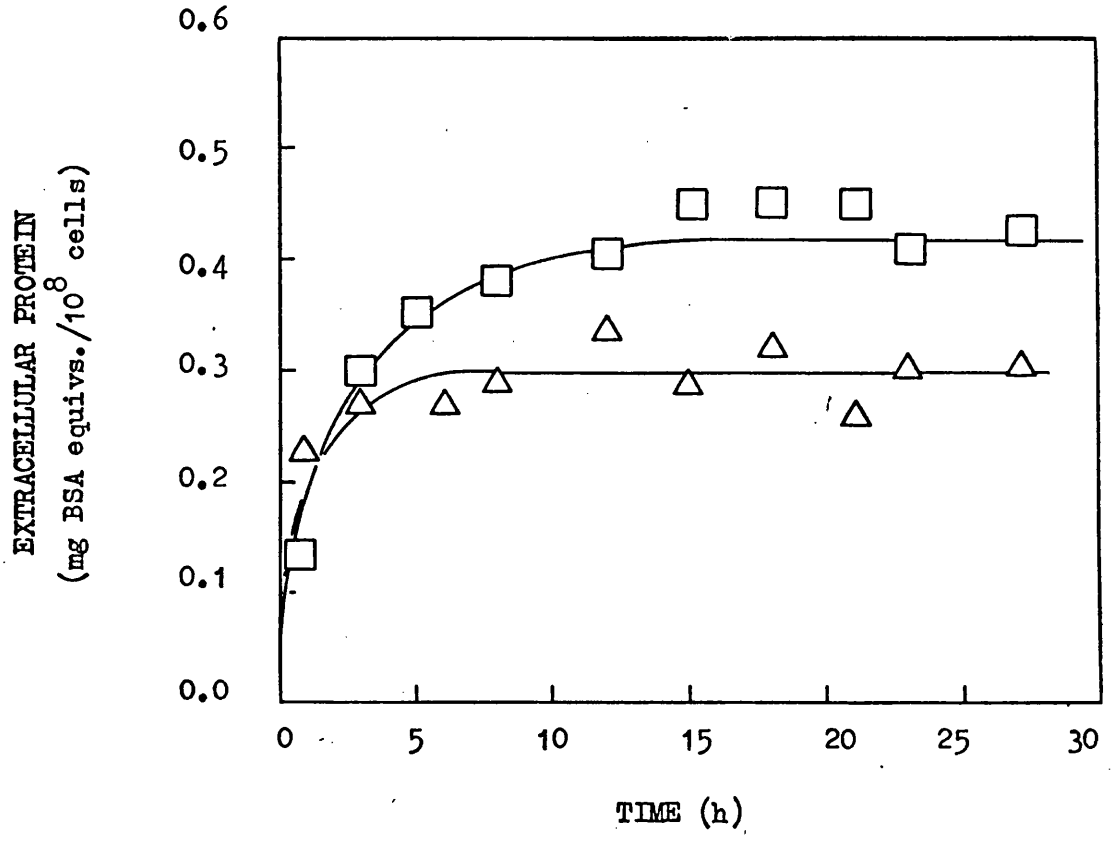


FIG. 38 Cellular Excretion of Protein during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.31mg glycogen/10^8 cells

△ ; myxamoebae initially contained 6.67mg glycogen/10^8 cells

Extracellular Amino Acids

Attempts were made to estimate the amounts of amino acids excreted during development using the ninnydrin assay (Mitchell & Moyle, 1953) and correcting for the reaction of ammonia with ninhydrin by the glutamic dehydrogenase method of Levitzki (1971). However, the large amounts of ammonia in the PDF prevented accurate and reproducible estimations of excreted amino acids. Thus, in order to determine whether the presence of high levels of glycogen in myxamoebae spares amino acid oxidation during development by enhancing excretion of amino acids, myxamoebae containing protein labelled with (U-^{14}C)-aspartate were allowed to develop and the excretion of radioactive material into the PDF was monitored. The results of this study showed that myxamoebae excrete similar amounts of radioactive material during development, irrespective of cellular glycogen content (Table 21).

Production of Ammonia During Development

Axenically-grown cells excrete large quantities of ammonia during development, excretion occurring immediately the cells are deposited on Millipore filter supports and continuing at a linear rate until culmination (Fig. 39). High levels of intracellular glycogen appear to have little effect on either the rate or extent of ammonia excretion (Fig. 39, Table 22).

Production of Carbon Dioxide During Development

It has already been demonstrated that myxamoebal glycogen is

TABLE 21 The relationship between myxamoebal glycogen content and the percentage of cellular protein excreted during development

Myxamoebal glycogen content ($mg/10^8$ cells)	Percentage of cellular radioactive protein excreted into the PDF during the 0-90 h period of development
0.26	4.27
2.81	3.66

Data shown in the above table were taken from Table 23 (b)(see later)

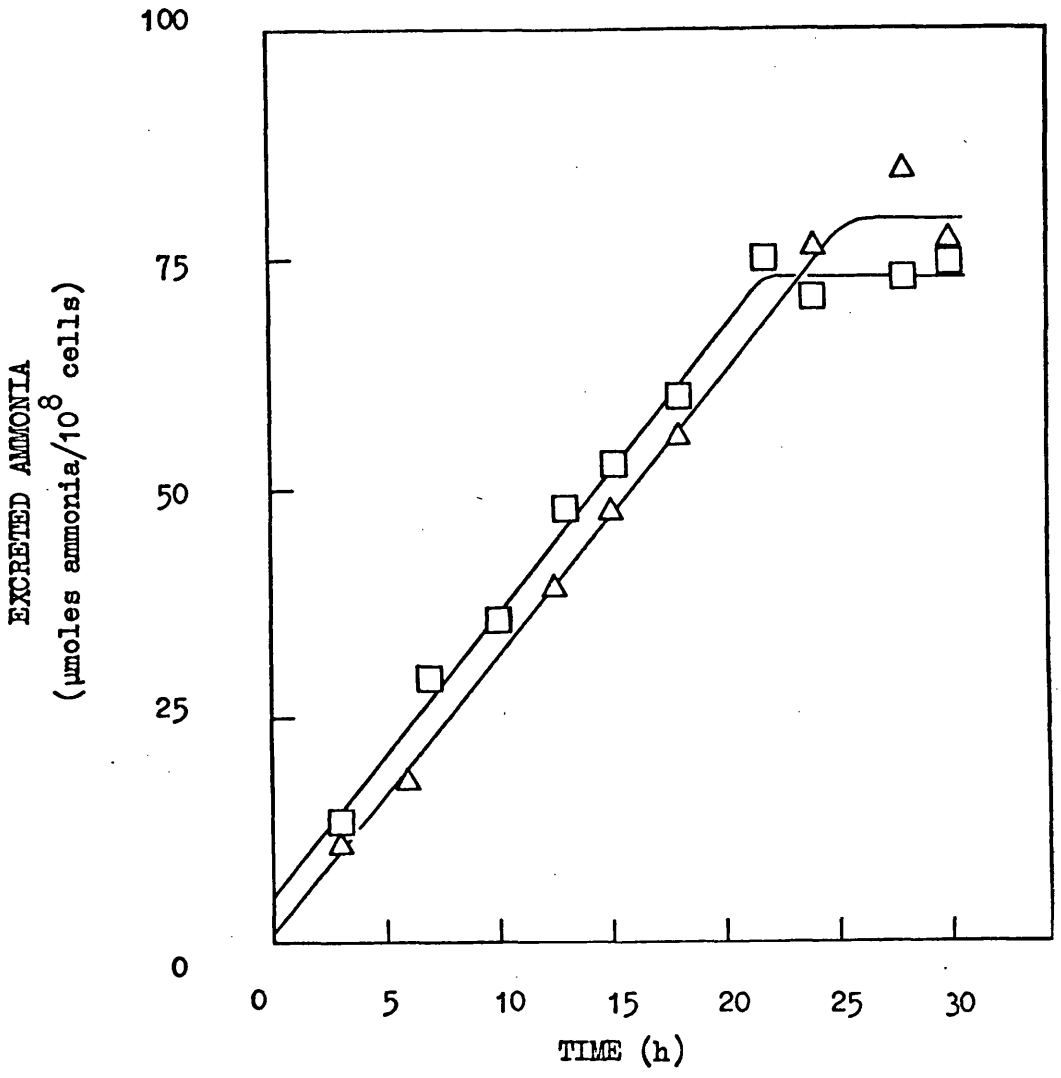


FIG.39 Cellular Ammonia Excretion during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.17mg glycogen/10^8 cells

△ ; myxamoebae initially contained 2.45mg glycogen/10^8 cells

TABLE 22 The relationship between myxamoebal glycogen content and the rate and extent of ammonia excretion during the developmental phase

Myxamoebal glycogen content ($mg/10^8$ cells)	Rate of ammonia excretion ($\mu moles/10^8$ cells/h)	*Percentage rate	Amount of ammonia excreted ($\mu moles/10^7$ cells)	**Percentage amount
0.060 ± 0.014 (2)	3.2019 ± 0.006 (2)	100.00	78.45 ± 4.33 (2)	100.00
0.171 ± 0.001 (2)	3.1834 ± 0.022 (2)	99.42	67.89 ± 0.21 (2)	86.54
0.320 ± 0.000 (2)	2.7282 ± 0.010 (2)	85.21	62.57 ± 1.02 (2)	79.76
1.230 (1)	2.5697 (1)	80.26	54.57 (1)	69.56
2.45 - 0.120 (3)	2.4573 - 0.2605 (3)	76.75	62.68 - 5.16 (3)	79.90

Results are given as means $\pm$ S.E.M. Figures in brackets refer to the number of experiments.

$$\text{* Percentage rate} = \frac{\text{Rate of ammonia excretion during the development of myxamoebae}}{\text{Rate of ammonia excretion during the development of myxamoebae initially containing } 0.060 \pm 0.014 \text{ (2) mg glycogen}/10^8 \text{ cells}} \times 100\%$$

$$\text{**Percentage amount} = \frac{\text{Amount of ammonia excreted during the development of myxamoebae}}{\text{Amount of ammonia excreted during the development of myxamoebae initially containing } 0.060 \pm 0.014 \text{ (2) mg glycogen}/10^8 \text{ cells}} \times 100\%$$

The discrepancy between corresponding figures in the percentage rate and percentage amount columns is caused by

(1) differences in the exact period of time over which ammonia is excreted (see Fig. 39)

(2) inaccuracy of the figure for the amount of ammonia excreted due to extrapolation to zero time in order to obtain a background ammonia value (see Fig. 39).

oxidised to carbon dioxide during development (Chapter II). Therefore, in order to follow the production of carbon dioxide from protein, cells were labelled with (U-^{14}C)-aspartate such that most of the label enters protein and not glycogen. Oxidation of protein to carbon dioxide was then followed by trapping ^{14}C-carbon dioxide in sodium hydroxide and determination of the radioactive content of this alkali.
This study revealed that most of the cellular protein degraded during development is oxidised to carbon dioxide* and, moreover, that the oxidation of up to 2.81 mg glycogen/10^8 cells has no significant effect on the extent of this protein oxidation (Table 23 a, b).

Cellular RNA Metabolism during Development

Cellular RNA decays rapidly from the onset of development, according to first order kinetics (Fig. 40).

Thus if: -

R_o = cellular RNA content per 10^8 cells at time 0

R_t = cellular RNA content per 10^8 cells at time t

t = hours of development

and if the reaction obeys first order kinetics,

$$\log_{10} \frac{R_t}{R_o} = -\frac{k}{2.303} t$$

such that a plot of $\log_{10} \frac{R_t}{R_o}$ against t yields a straight line,

slope $-\frac{k}{2.303}$, where k is the first order rate constant (Fig.41).

*That the gas trapped in the alkali was mainly carbon dioxide was demonstrated by mass spectrometry (see Appendix B).

TABLE 23 (a) The oxidation of myxamoebal protein to carbon dioxide during the developmental phase

Sample (material and developmental time)	Total Radioactivity (cpm/10^8 cells)		Specific Radioactivity (cpm/μg atom carbon)	
	*NS	*G	*NS	*G
Total cells				
0 h	44,735	17,494	-	-
90 h	16,388	5,194	-	-
Cellular Glycogen				
0 h	3,333	1,479	387	16
90 h	944	240	494	98
Cellular protein				
0 h	31,000**	13,700**	48	26
90 h	6,750**	2,500**	44	22
Carbon dioxide 90h	31,360	13,120	-	-
PDF 90h	1,910	640	-	-
Sorocarp wash water 90h	Counts little above background			

Figures are the means of three determinations.

*NS: myxamoebae initially containing 0.26 mg glycogen/10^8 cells

*G : myxamoebae initially containing 2.81 mg glycogen/10^8 cells

** These figures are estimates based on:

(1) the specific radioactivity of TCA insoluble protein

(2) the total protein content of cell extracts, and thus do not take account of the specific radioactivity of cellular TCA soluble protein.

(3) the assumption that the average molecular weight of the amino acids of D. discoideum protein is 100.

TABLE 23 (b) The oxidation of myxamoebal protein to carbon dioxide during the developmental phase

Sample	Total radioactivity lost or gained over 90 h period of development ($cpm/10^8$ cells)		Total radioactivity lost or gained as a percentage of total cell radioactivity lost over 0-90 h period of development	
	*NS	*G	*NS	*G
LOSS				
Total cells	28,347	12,300	100.00	100.00
Cellular glycogen	2,389	1,239	8.48	10.08
Cellular protein	24,250**	11,120**	85.70	90.50
GAIN				
Carbon dioxide	31,360	13,120	110.50	106.90
PDF	1,910	640	4.27	3.66
Sorocarp wash water	Negligible			

*NS: myxamoebae initially containing 0.26 mg glycogen/10^8 cells

*G : myxamoebae initially containing 2.81 mg glycogen/10^8 cells

** These figures are estimates (see Table 23 (a))

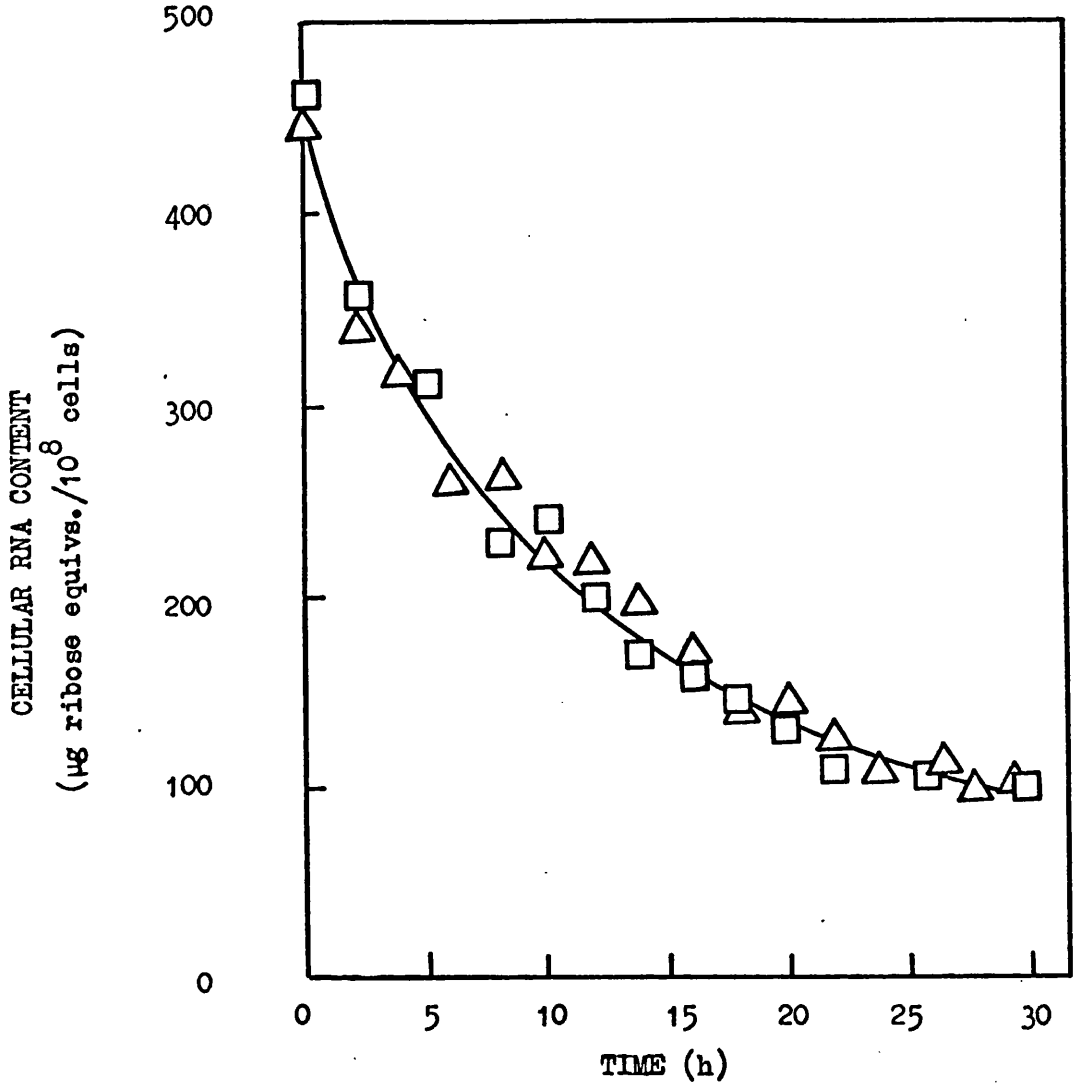


FIG.40 Changes in The Cellular RNA Content during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.094mg glycogen/10^8 cells

△ ; myxamoebae initially contained 1.112mg glycogen/10^8 cells

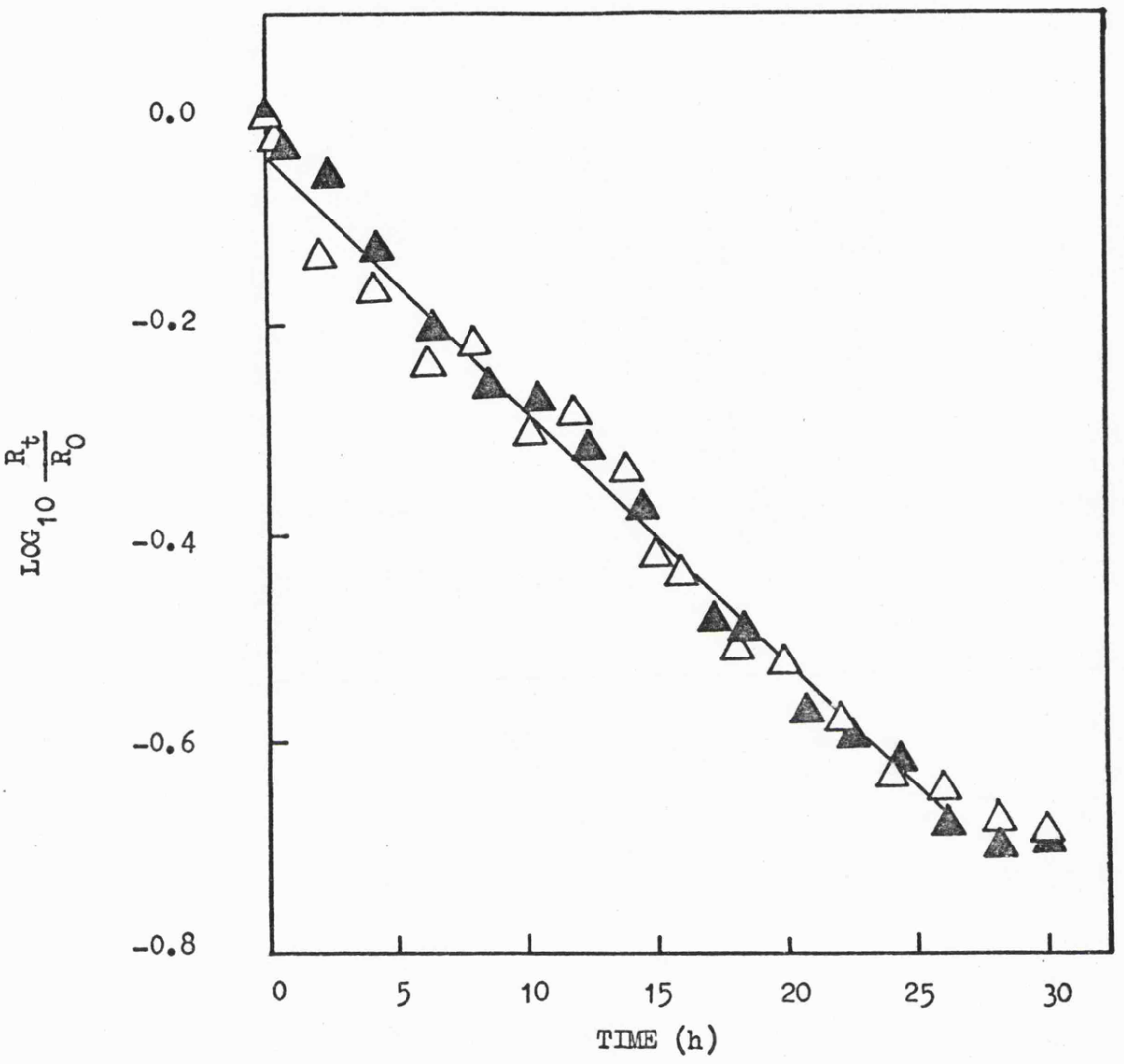


FIG. 41 First Order Kinetic Plot of RNA Degradation Data.

Two separate experiments are shown using myxamoebae of similar glycogen contents:-

△ ; myxamoebae initially contained 1.24mg glycogen/10^8 cells

▲ ; myxamoebae initially contained 1.11mg glycogen/10^8 cells

For explanation of this plot see text.

Values of k, determined for myxamoebae initially containing various levels of glycogen are shown in Table 24, and indicate that the rate of cellular RNA degradation is unaffected by the quantity of myxamoebal glycogen present.

A puzzling observation is the variability of cellular RNA content and the amount of RNA degraded during development (Table 24). The variability of similar data obtained by White & Sussman (1961) using bacterially-grown myxamoebae was explained by Hanks (1967) as being due to differences in the stage of growth of the myxamoebae used. In our experiments there appears to be no correlation between the amount of myxamoebal RNA present and / or degraded during development, and the phase of vegetative growth at which the cells were harvested. It is possible that the variation observed in myxamoebal RNA content is due to variation in the components of the growth medium from batch to batch, as found for myxamoebal glycogen contents of cells grown in axenic medium in the absence of added glucose (Chapter I), although there is no direct evidence for this. However, since both the vegetative myxamoebal RNA level and the amount of RNA degraded during development vary considerably yet the rate of RNA degradation and the sorocarp RNA content are fairly constant from experiment to experiment (Table 24), this may suggest that much of the variability is due to the extremely rapid rate of RNA degradation following removal of nutrients. (Fig. 40). Relatively minor differences in time taken to harvest cells and take the vegetative cell samples would be expected to result in fairly large differences in observed vegetative myxamoebal RNA content

TABLE 24: The relationship between myxamoebal glycogen content and the rate and extent of RNA degradation during the developmental phase.

Cellular Glycogen content (mg/10^8 cells)	Myxamoebal RNA content (μg_8 ribose equivs. /10^8 cells)	Sorocarp (30h.) RNA content (μg_8 ribose equivs. /10^8 cells)	Amount of RNA degraded during the 0-30h. period of development (μg_8 ribose equivs. /10^8 cells)	Rate of RNA degradation (k in h^{-1})	Percentage of Myxamoebal RNA degraded during the 0-30h period of development (μg ribose equivs./10^8 cells)	*Percentage Rate	*Percentage Amount
0.071±0.014 (3)	302±29 (3)	62±4 (3)	240±27 (3)	0.0511±0.0041 (3)	79.2	100.00	100.00
0.321±0.000 (2)	484±28 (2)	103±7 (2)	349±11 (2)	0.0592±0.0028 (2)	78.9	115.85	145.40
1.193±0.041 (3)	371±39 (3)	88±10 (3)	256±39 (3)	0.0545±0.0018 (3)	76.1	106.65	106.70
1.736±0.244 (2)	284±20 (2)	79±11 (2)	204±9 (2)	0.0502±0.002 (2)	72.3	98.24	85.00
2.670 (1)	265 (1)	90 (1)	175 (1)	0.0481 (1)	66.0	94.13	72.92
3.620 (1)	418 (1)	105 (1)	313 (1)	0.0440 (1)	75.0	86.11	130.40
5.560 (1)	276 (1)	84 (1)	192 (1)	0.0451 (1)	69.5	88.26	80.00

Results are given as means±S.E.M.

Figures in brackets refer to the number of experiments.

Each rate of RNA degradation (calculated in terms of k, the first order rate constant, as described in the text) was calculated using 10 experimental points, on average; range 6-14.

$$\text{Percentage Rate} = \frac{\text{Rate of RNA degradation by myxamoebae during development}}{\text{Rate of RNA degradation by myxamoebae initially containing } 0.071\pm0.014\ (3)\ \text{mg glycogen}/10^8\ \text{cells.}} \times 100\%$$

$$\text{Percentage Amount} = \frac{\text{Amount of RNA degraded by myxamoebae during the 0-30h period of development}}{\text{Amount of RNA degraded by myxamoebae initially containing } 0.071\pm0.014\ (3)\ \text{mg glycogen}/10^8\ \text{cells during the 0-30h period of development.}} \times 100\%$$

and hence in the amount of RNA degraded during development. In any case, it is clear that the rate of RNA degradation is unaffected by the oxidation of large amounts of myxamoebal glycogen (Table 24).

Intracellular TCA-soluble Orcinol-positive Material

Since RNA is rapidly degraded during development it was of interest to determine the fate of the degradation products. Thus the utilisation of glycogen as an energy source could possibly prevent the use of RNA degradation products as energy source whilst allowing RNA degradation to proceed unaffected.

Measurement of TCA-soluble orcinol-positive material indicates that soluble pentose intermediates do not accumulate during development but rather decay rapidly (Fig. 42). The myxamoebal pool of TCA-soluble orcinol-positive material is fairly variable in size but during development decays to approximately 20 µg ribose equivalents/10^8 cells at 25 h. developmental time irrespective of initial pool size or myxamoebal glycogen content (Fig. 42). [It should be noted that not all the material present in the TCA-soluble fraction and reacting with the orcinol reagent is ribose since other sugars, for example glucose, also react to some extent (Mejbaum, 1939).]

Extracellular Orcinol-positive Material

During development, orcinol-positive material is excreted by the cells. However, only low levels are extruded compared to the amount of RNA degraded and the amount excreted does not vary significantly with changes in the myxamoebal glycogen content (Fig. 43).

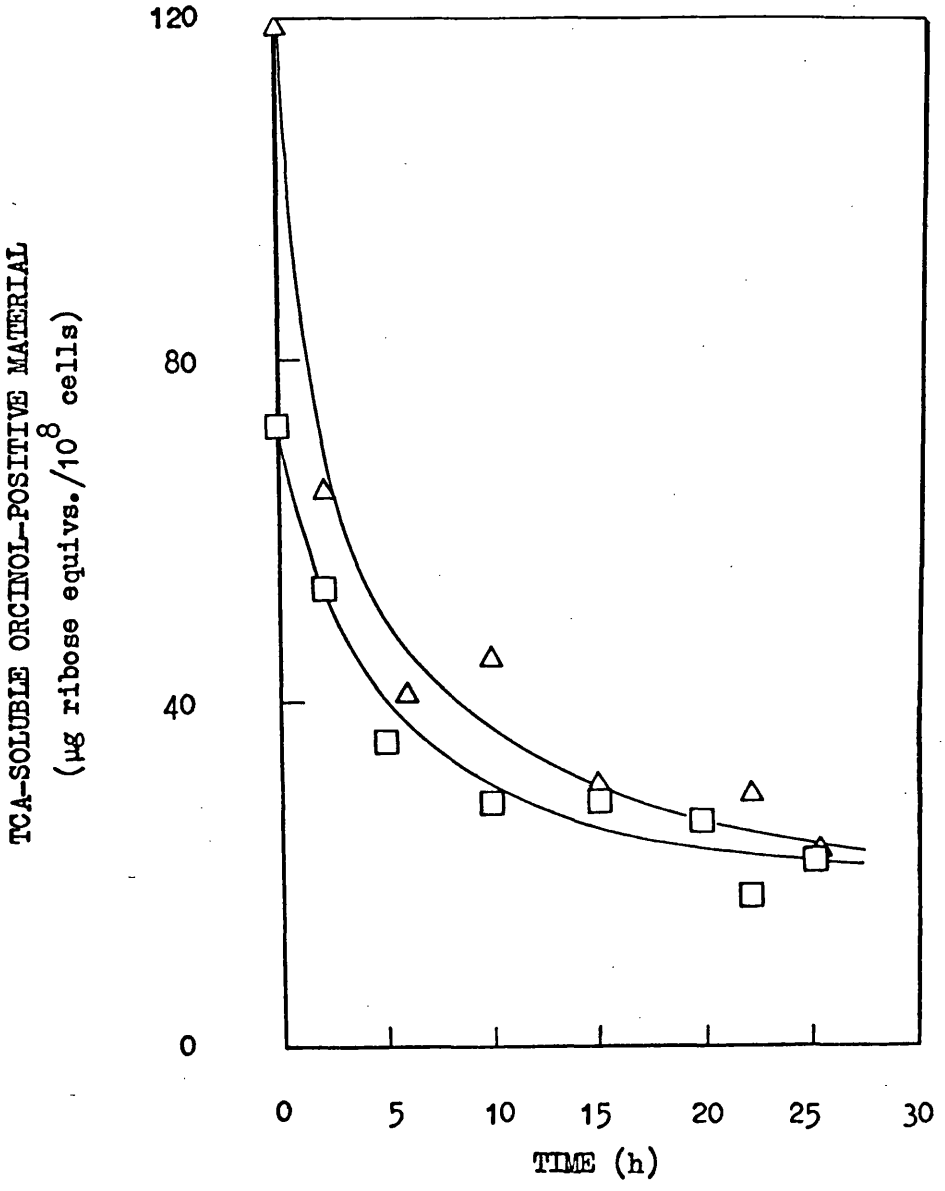


FIG. 42 The Decay of Cellular TCA-soluble Orcinol-positive Material during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.26mg glycogen/10^8 cells

△ ; myxamoebae initially contained 3.62mg glycogen/10^8 cells

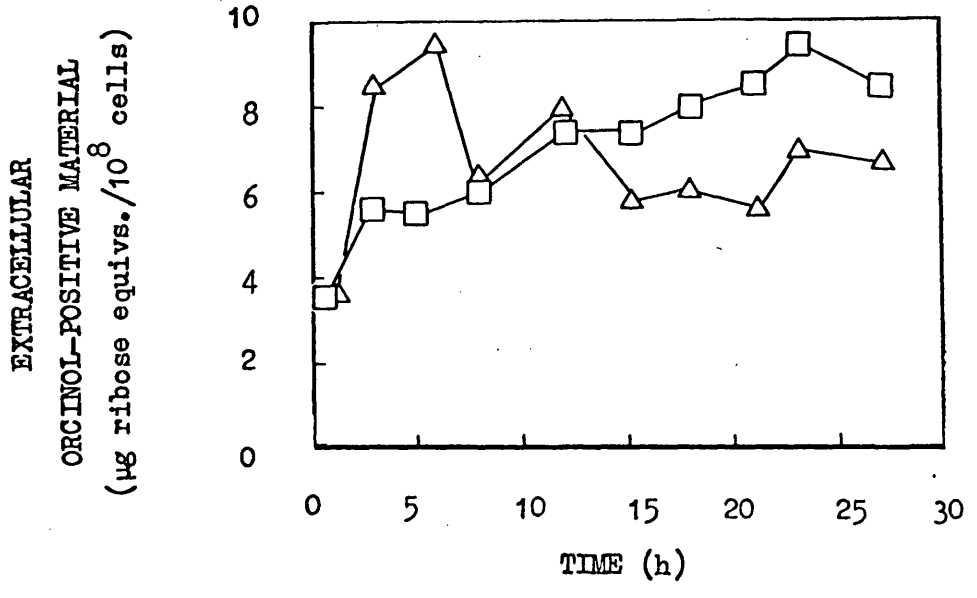


FIG.43 Cellular Excretion of Orcinol-positive Material during The Development of Axenically-grown Myxamoebae.

□ ; myxamoebae initially contained 0.29mg glycogen/10^8 cells

△ ; myxamoebae initially contained 6.77mg glycogen/10^8 cells

Extracellular U.V. Light Absorbing Compounds

Hanks (1967) observed that during the first few hours of development of myxamoebae grown on bacteria, cellular RNA was rapidly degraded and RNA bases, after a few hours of retention, were lost from the cells. The data in Fig. 44(a,b) show that axenically-grown cells excrete U.V. light absorbing compounds but that the excretion follows the same time course as excretion of Folin-positive material (see Fig.38). Moreover, the O.D. $\frac{260}{280}$ ratio increases only slightly or not at all (depending on the experiment) during the first three hours of development and then remains constant (Fig. 44c). These data are incompatible with RNA degradation, retention of RNA bases in the cells and their subsequent excretion after several hours of development.

FIG.44 Cellular Excretion of Ultra-Violet Light Absorbing Material during The Development of Axenically-grown Myxamoebae.

Fig.44(a)

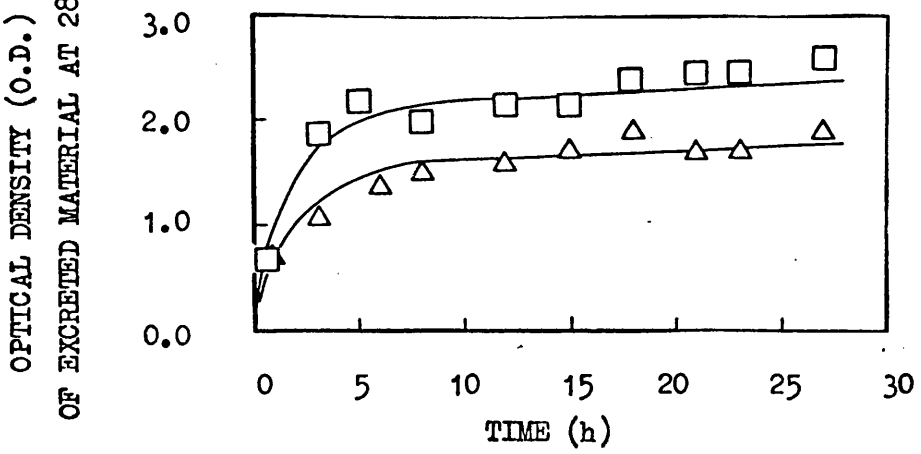


Fig.44(b)

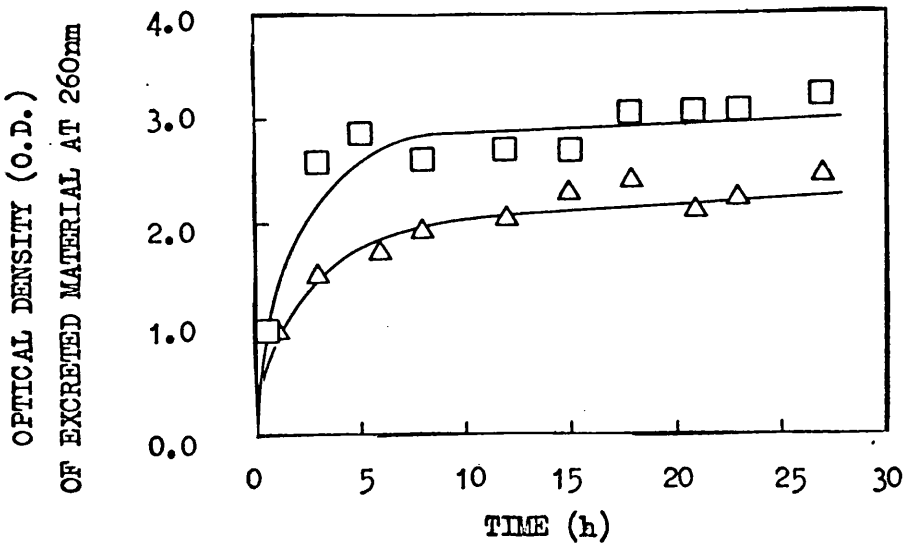
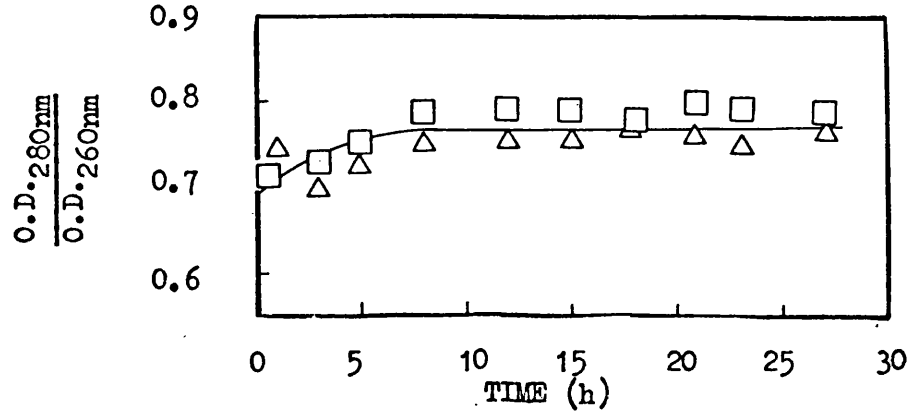


Fig.44(c)



□; myxamoebae initially contained 0.29mg glycogen/10^8 cells

△; myxamoebae initially contained 6.77mg glycogen/10^8 cells

DISCUSSION

It is perhaps a little surprising and certainly rather depressing that it is now eleven years since it was discovered that the majority of cellular protein and RNA is degraded during the development of *D. discoideum* (White & Sussman, 1961), and yet we still cannot state with certainty the fate of these major cell constituents.

The Metabolism of Protein During Development

The exact picture of protein metabolism during the development of bacterially-grown myxamoebae is controversial: -

(1) Gregg *et al* (1954) reported little protein breakdown during the transition from vegetative myxamoebae to the migrating slug stage but a rapid decrease from the latter stage to fruiting body construction.

(2) Wright and Anderson (1960) reported a 70% drop in the amino acid pool levelling off at late migration, a 40% fall in ethanol soluble protein levelling off at late migration, and a 20% decrease in ethanol insoluble protein starting at late migration and continuing throughout the rest of development.

(3) White & Sussman (1961) found that the total protein content of bacterially-grown myxamoebae varied between approximately 2-6 mg per 10^8 cells but that during development degradation resulted in a fruiting body protein content of approximately 2-2.5 mg per 10^8 cells.

Fractionation revealed that during development TCA-soluble protein rapidly decreased by 80% and then levelled off at late migration whilst TCA-insoluble - alcohol-soluble, alcohol-insoluble - hot TCA-soluble and alcohol-insoluble - TCA-insoluble protein fractions all decreased more slowly than TCA-soluble protein to about 30% off their myxamoebal level and with the same decay kinetics as total protein. Unlike TCA-soluble protein, the other protein fractions decayed continually throughout development and only stabilised after fruiting body construction.

(4) Hanks (1967), studying what he termed the 'interphase' period of development (the first 6-7 h of development before aggregation) determined that TCA-soluble protein increased by approximately 40% during the first few hours of development and then decreased, whilst TCA-insoluble - alcohol-insoluble protein decreased continually from the onset of the developmental phase.

(5) Mizukami & Iwabuchi (1970), using myxamoebae grown on dead bacteria in liquid culture, reported total cellular protein to decrease to approximately 50% of the vegetative myxamoebal value, degradation occurring throughout development.

These results are therefore unanimous in that protein is degraded during development but disagree as to the extent and timing of degradation the various protein fractions.All the above studies except that of

Hanks (1967) and Mizukami & Iwabuchi (1970) employed myxamoebae grown on bacterial lawns and harvested when nearly all the bacteria had been ingested. As pointed out by Hanks (1967), this method is likely to yield myxamoebae at various stages of starvation from experiment to experiment (and worker to worker) and so tend to give irreproducible results. In support of this idea, Hanks (1967) found that the variability of myxamoebal protein content observed by White & Sussman (1961) could be eliminated by growing the cells on live bacteria in liquid culture and harvesting in the exponential phase of growth, when they contain approximately 6 mg protein per 10^8 cells. However, no reason has been found to explain the other conflicting data; for example, why Wright & Anderson (1960) observed alcohol-insoluble protein to remain undegraded until late migration whilst White & Sussman (1961) and Hanks (1967) observed a rapid decrease in this fraction from the start of development, although contamination of the developing cells with bacteria to different extents could be part of the answer. As stated by Inselburg & Sussman (1967) "At present, numerous developmental studies are being made with phagotrophs like the slime moulds and ciliated protozoa and with forms like sea urchins, sponges and Acetabularia which are contaminated with bacterial parasites and symbionts. The danger that the bacteria contribute to the data is always serious."

The use of myxamoebae grown axenically in liquid culture has the advantage that Hanks (1967) found using shake cultures, namely, that cells can be obtained at a reproducible stage of growth merely by harvesting at the desired cell density, and the added advantage that the

developing system is free of biochemical contamination by bacteria.

The data presented in this chapter show that axenically-grown myxamoebae routinely contain approximately 10-12 mg protein per 10^8 cells when harvested at any time in the exponential phase of growth or early stationary phase in agreement with the results of Ashworth & Watts (1972). Protein degradation begins at the onset of development; TCA-soluble protein and the amino acid pool decrease very quickly, with similar kinetics, reaching low levels during the slug migration stage and remaining there throughout the rest of development. At no time was an increase observed in this fraction in contradiction to the results of Hanks (1967) but in agreement with the work of White & Sussman (1961) and Wright & Anderson (1960a). The cellular content of TCA-insoluble protein drops suddenly at the beginning of development and then decreases more slowly than TCA-soluble protein with linear kinetics, degradation proceeding until fruiting body formation, in agreement with the results of White & Sussman (1961) and Hanks (1967) but contrary to the results of Wright & Anderson (1960) and Gregg et al (1954). At least part of the initial rapid decrease in TCA-insoluble protein, and probably some of the decrease in TCA-soluble protein, appears to be due to cellular excretion of Folin-positive material, but after the first few hours of development this excretion ceases or continues only at a very low rate.

In conclusion, apart from the data of Hanks (1967), it appears that in both bacterially and axenically-grown myxamoebae, the TCA-soluble pool of proteins and the amino acid pool are rapidly metabolised from the onset of development and at a much faster rate than the TCA-insoluble proteins, reaching basal levels during slug migration

whilst most TCA-insoluble proteins, once degradation has commenced, are metabolised throughout development.

In starving E.coli (Mandelstam, 1958) and yeast (Halvorson, 1958) the amino acid pool also decreases very early, being rapidly incorporated into cellular protein. This appears in part to be the fate of the amino acid pool during the early phases of D. discoideum development (Wright & Anderson, 1960b). There is also some evidence that amino acids and ammonia may suppress proteolysis which would therefore be activated by an initial decrease in amino acid pool size (Mandelstam, 1960; Levisohn & Aronson, 1967). It is therefore clear that extensive proteolysis occurs during the development of D. discoideum, but for what purpose?

As early as 1954, Gregg et al discovered that during the developmental phase, myxamoebae of D. discoideum degrade protein and excrete ammonia, and Wright (1963) has shown that developing cells are capable of metabolising glutamate to carbon dioxide, probably via glutamate dehydrogenase. These data suggest that developing cells of D. discoideum degrade their protein to products which may then be oxidised to carbon dioxide and ammonia; a conclusion that is confirmed by the results described in this chapter showing that axenically-growing cells degrade approximately 7-9 mg protein and produce about 60-80 umoles of ammonia during development (Table 25) roughly the amount of ammonia one would expect to be produced if most of the degraded protein was oxidised. Moreover, myxamoebae whose protein has been labelled as a consequence of growth in axenic medium containing (U-^{14}C)-aspartate,

TABLE 25 Comparison of the amount of myxamoebal protein degraded and the amount of ammonia excreted during the development of axenically-grown myxamoebae initially containing various amounts of glycogen

Myxamoebal glycogen content (mg/10^8 cells)	Amount of myxamoebal protein degraded during the 0-26 h period of development (mg/10^8 cells)	Amount of ammonia excreted during development (μmoles/10^8 cells)
0.071, 0.060	8.43 $\pm$ 0.26 (3)	78.45 $\pm$ 4.33 (2)
0.321, 0.320	8.64 $\pm$ 0.46 (2)	62.57 $\pm$ 1.02 (2)
1.176, 1.230	7.07 $\pm$ 0.12 (2)	54.57 (1)
2.670, 2.450	6.83 (1)	62.68 $\pm$ 5.16 (3)

Results are given as means $\pm$ S.E.M.

Figures in brackets refer to the number of experiments

* This table is a collection of data from Tables 20 and 22. Thus, the corresponding figures for ammonia production and protein degradation do not relate to the same set of cells and, for this reason, each myxamoebal glycogen content is denoted as two figures, the first figure is the mean glycogen content of myxamoebae used for protein degradation studies, the second is the mean glycogen content of myxamoebae used for ammonia production studies.

vent most of the labelled carbon as ^{14}C-oarbon dioxide. This is good evidence that the majority of degraded myxamoebal protein is oxidised to carbon dioxide and ammonia, presumably yielding energy. The available data therefore supports the view that protein acts as a source of energy during development, but this does not mean that energy production is the sole or even the main reason for developmental protein degradation, a point that will be discussed in detail later.

It is interesting that the rate of ammonia excretion is linear throughout development of axenically-grown cells. This raises important doubts as to the significance of the marked increases in L-tyrosine transaminase, L-alanine transaminase, L-serine dehydrase and L-threonine dehydrase activity observed during the developmental phase (see Pong & Loomis, 1971 for refs.). These enzymes are extremely important in the interconversion and de-amination of amino acids yet it appears that increasing their amount in the cell has no effect upon the rate of ammonia production, suggesting that the limiting factor in amino acid oxidation is not cellular enzyme content but substrate availability; possibly one more example of the general contention that "changes in the levels of certain essential enzymes are not significant with respect to controlling the metabolite flux necessary for the accumulation of specialised end-products." (Wright, 1970).

It should be noted that Wright (1963) found that the *in vivo* rate of glutamate oxidation increases five-fold from pre-culmination to the mature sorocarp stage, whereas according to the data presented in this chapter, the rate of ammonia production is constant or even decreasing (Fig. 39). This discrepancy has not been explained.

The Metabolism of RNA During Development

White & Sussman (1961) determined that the total RNA content of bacterially-grown myxamoebae is approximately 1 mg/10^8 cells, but Hanks (1967) found 2 mg RNA/10^8 cells, and Watts & Ashworth (1971) observed intermediate values. These discrepancies are probably in part due to the different myxamoebal growth conditions used since White & Sussman (1961) and Watts & Ashworth (1971) grew cells on bacterial lawns whereas Hanks (1967) grew cells on live bacteria in liquid culture.

Studying the development of bacterially-grown myxamoebae, Krivanek (1964) found a four-fold increase in total cellular RNA during the transition from vegetative myxamoebae to the migrating slug stage, and a decrease in RNA from the latter stage to sorocarp construction back to the original myxamoebal RNA level.

In direct contradiction to these results, the total RNA content of such cells has been observed to decrease from the onset of development (White & Sussman, 1961; Pannbacker, 1966; Hanks, 1967; Mizukami & Iwabuchi, 1970), degradation continuing through fruiting body construction such that about 70% of cellular RNA is lost (White & Sussman, 1961). The latter workers attributed the decrease of RNA early in development to the final digestion of ingested bacteria, but Hanks (1967) concluded that the decrease observed is due to the breakdown of amoebal RNA.

The study of RNA metabolism during the development of axenically-grown myxamoebae, as described in this chapter, is important in this respect, since it represents the first study of this kind in

the absence of bacterial contamination. The data obtained show that axenically-grown myxamoebae contain approximately 1-2 mg total cellular RNA, in agreement with the data of Watts & Ashworth (1971) and that this RNA is rapidly degraded from the start of development according to first order kinetics, degradation continuing throughout fruiting body construction, and resulting in an approximately 70% loss of cellular RNA. The pattern of RNA metabolism observed during the development of axenically-grown myxamoebae is therefore very similar to that observed by most workers (White & Sussman, 1961; Pannbacker, 1966; Hanks, 1967; Mizukami & Iwabuchi, 1970) during the development of bacterially-grown cells, indicating that the rapid RNA degradation that occurs during the development of bacterially-grown cells is probably due to degradation of myxamoebal RNA as suggested by Hanks (1967) and not to final digestion of ingested bacteria as proposed by White & Sussman (1961). The majority of RNA degraded appears to be ribosomal (Hanks, 1967; Cocucci & Sussman, 1970).

What is the purpose of RNA degradation during development? One approach to an answer is to determine the fate of degraded myxamoebal RNA.

Using (^{3}H)-uridine labelled D. discoideum myxamoebae, Hanks (1967) found that RNA was degraded during development but that no large molecular weight degradation products were observed. Nor was there an accumulation of low molecular weight material in the 4S region of sucrose density gradients. However, label did accumulate in the TCA-soluble fraction, although no ribose (estimated as orcinol-positive material) accumulated here. After a few hours, label was lost from

the cells. Therefore, Hanks (1967) suggested that during development of D. discoideum, RNA is degraded to its constituent nucleotides "which are then split to bases plus ribose; the ribose is metabolised as an energy source, the bases accumulate in the amoebae for a few hours before being lost from the cells." This is supported by the work of Sussman (1967) which strongly suggested that during the development of Polysphondylium pallidum, another cellular slime mould, RNA is degraded to products which are lost from the cells and not incorporated into cellular components (other than some newly-synthesised RNA).

Such a pattern of RNA degradation is not without precedent. Comb & Brown (1964) reported a loss of ribosomal RNA during the development of sea urchin, and later, Slater & Spiegelman (1966) were unable to detect the degradation products in sucrose gradients. During the starvation of bacteria, Dawes & Ribbons (1965) showed that following RNA degradation, ribose was oxidised whilst U.V.-absorbing material was released to the medium. But is this really the fate of degraded RNA during the development of D. discoideum? Let us consider the fate of the RNA bases. Although Hanks (1967) showed that labelled bases from degraded radioactive myxamoebal RNA entered a TCA-soluble pool and then, after a few hours, were lost from the cells, he did not attempt to locate the lost label, but it can be assumed that either labelled compound(s) were excreted into the extracellular fluid and/or lost as gas(es).

Now, although RNA is degraded throughout the developmental phase of axenically-grown myxamoebae, the majority of U.V.-absorbing material that is excreted is excreted during the first 4-5 hours of

development. After this time, little or no further excretion occurs. This argues against a cellular retention of RNA bases during the first few hours of development followed by their excretion, unless the excretion of protein and amino acids (U.V.-absorbing materials) early in development masks a slightly later excretion of RNA bases. That this is not so is shown by the fact that the O.D. $\frac{260}{280}$ ratio remains constant or rises slightly during the first few hours of development (depending on the experiment) and then remains constant during the rest of development. If RNA bases were excreted directly into the extracellular fluid one would expect to observe an increase in this ratio at the appropriate time and this is clearly not so. Also, there are indications that the excretion of U.V.-absorbing material that occurs during development is not a consequence of development itself but rather a consequence of cell suspension in an unfavourable medium such that the cells initially leak cellular material but, as development proceeds, recover permeability barriers and cease excretion. Thus, Krichevsky & Love (1965) showed that vegetative D. discoideum myxamoebae, when suspended in distilled water, excrete RNA, protein and low molecular weight ninhydrin-positive materials (possibly amino acids) and that the ability of compounds to stimulate the overall rate of morphogenesis is well correlated with the prevention of cellular leakage of these compounds. The available evidence therefore argues against a massive excretion of RNA bases from the cells following RNA degradation.

The only alternative fate is that the bases are metabolised intracellularly and then the non-U.V.-absorbing product(s) are excreted into the extracellular fluid and/or vented as gas(es). There is some

evidence from studies of the degradation of RNA during the starvation of bacteria that the bases may be metabolised further to yield ammonia as one product and carbon dioxide as another (Stephenson & Trim, 1938; Burleigh et al, 1963). This could also occur during the development of D. discoideum since both ammonia and carbon dioxide are known to be evolved during this phase and although ammonia is trapped in the acidic PDF, it does not absorb light strongly in the U.V. region. This metabolic scheme also accounts nicely for the loss of cellular label from ^{3}H-uridine-labelled (Hanks, 1967) or ^{14}C-uridine labelled cells (Sussman, R.R., 1967) without incorporation into non-nucleic acid cell components or excretion of bases.

Alternatively, the nitrogen of the bases may be vented as ammonia and the carbon skeleton not oxidised but excreted into the extracellular fluid. However, this is more unlikely since the excretion of other cellular reserves occurs during the first few hours of development whereas RNA degradation and base metabolism must occur throughout this phase, and secondly, the excretion which does occur appears to be the result of experimental technique rather than of developmental significance (see above).

Whilst it is obvious that the fate of bases from degraded D. discoideum RNA is not known with any certainty, it is clear from the results presented in this chapter that they are not accumulated within the cell, nor excreted in large quantities into the extracellular fluid. Rather they are metabolised intracellularly and may be oxidised as an energy source.

Let us now consider the fate of ribose from degraded myxamoebal RNA.

Although axenically-grown myxamoebae degrade approximately 70% of their RNA during the developmental phase, no orcinol-positive material accumulates in the cells in agreement with the results of Hanks (1967). Nor is there a significant excretion of orcinol-positive material into the extracellular fluid. These data indicate that the ribose of degraded RNA is further metabolised by developing cells of D. discoideum, possibly to carbon dioxide, [although this is difficult to prove because of the impracticability of labelling mainly RNA ribose and not other cellular sugars, and because carbon dioxide is produced from protein and glycogen oxidation anyway] and may therefore act as an energy source as suggested by Hanks (1967).

In summary, there is good evidence for believing that the products of RNA degradation are not excreted from developing cells of D. discoideum, unlike some other starving microbial systems (e.g. Dawes & Ribbons, 1965), but are further metabolised. The details of this metabolism are not known but there are no results which argue against the oxidation of RNA breakdown products as energy sources except that some RNA nucleotides are probably used for the synthesis of new RNA which occurs during development.

The Interrelationship between the Utilisation of Glycogen, Protein and RNA as Energy Sources

The main questions this chapter attempts to answer are:-

(1) Does glycogen act as an energy source during development?

(2) If so, what effect does this have on the use of protein and RNA during development?

In Chapter II, I showed that glycogen is rapidly degraded during development and the majority of products oxidised to carbon dioxide. Does this process yield metabolically useful energy?

In Chapter I, it was noted that myxamoebae containing high levels of glycogen aggregate much faster than those containing low levels, possibly suggesting that the former have access to a larger energy supply than the latter. Since the major difference in cellular reserves between the two sets of cells is the level of myxamoebal glycogen, this may indicate that glycogen can be degraded to yield metabolically useful energy. However, Liddel & Wright (1961) failed to show a stimulation of respiration by addition of exogenous glucose prior to slug formation, suggesting that the cells are not limited as regards energy supply at this time. In fact, such a result is not too surprising since bacterially-grown cells of D. discoideum as used by Liddel & Wright (1961) are largely impermeable to glucose early in development (Wright & Bloom, 1961) and therefore even if they were limited for energy, this could not be detected by the addition of exogenous glucose, whereas an internal supply of carbohydrate via glycogenolysis would be expected to stimulate respiration and possibly rate of development. Why the development of myxamoebae containing high levels of glycogen is not overall faster than myxamoebae containing low levels of glycogen is not clear especially since externally added glucose is reported to stimulate the formation of fruits (Krichevsky & Wright, 1963).

More compelling evidence that myxamoebal glycogen is metabolised as an energy source during development is provided by an analysis of the degradation of glycogen itself. In Chapter II, it was shown that myxamoebal glycogen is probably degraded during development by cellular amylases, of which β amylase probably predominates although α amylase may also be present. The products of this mixed amylase digestion would be maltose (α and β amylases) and glucose (α amylase) (Bernfeld, 1955), but since developing cells contain an active maltase (Chapter II), glucose would soon accumulate unless it is further metabolised. In fact, although the pool size of glucose is greater in developing cells initially containing 5.590 ± 0.048 mg glycogen/10^8 cells than in cells initially containing 0.308 ± 0.002 mg glycogen/10^8 cells, glucose does not accumulate until near the end of development, at which time the majority of myxamoebal glycogen has been degraded. Glucose is therefore utilised and the finding that G6P levels are also enhanced in developing cells containing high glycogen levels but that G6P does not accumulate until culmination strongly suggests that the metabolism of glucose occurs via G6P formation.

Some early work of Wright et al (1964) and Liddel & Wright (1961) is relevant here. These workers reported that when exogenous ^{14}C-glucose is supplied to D. discoideum at various stages of development, respiration is stimulated and the glucose is metabolised to ^{14}C-carbon dioxide. Increased levels of extracellular glucose, and by inference, increased levels of intracellular glucose, markedly influenced the *C6:C1 ratio from 0.3 to values greater than 1. Now, if glycolysis were

*The C6:C1 ratio is the ratio of the rate of carbon dioxide evolution from the C6 of glucose to the rate of carbon dioxide evolution from the C1 of glucose. The interpretation of C6:C1 ratios as regards the operation of various pathways, exchange reactions and recycling of intermediates is extremely complex, but it is accepted that C6:C1 ratios do give an indication of the metabolic pathways operative in utilising the glucose (Katz & Wood, 1960).

the main route of glucose metabolism one would expect a C6:C1 ratio of unity. Thus the glucose appears to be metabolised via other routes, possibly, as suggested by Gustafson & Wright (1972) via formation and oxidative decarboxylation of 6-phosphogluconate and via formation and oxidative decarboxylation of a uronic acid (galacturonic acid?), both yielding pentose phosphate as decarboxylation product. Utilisation of a hexose monophosphate shunt could then convert this back to hexose (Fig. 45). The net result would be to completely combust G6P to carbon dioxide with release of carbon exclusively from the 1 position (6-phosphogluconate pathway) or exclusively from the 6 position (uronic acid pathway) according to the stoichiometry: -

$$6 \text{ hexose-P} + 3\,O_2 + H_2O \longrightarrow 5 \text{ hexose-P} + 6\,CO_2 + 12[H] + P_i$$

(Gustafson & Wright, 1972)

However, the important point is that there is no known metabolic route in D. discoideum by which G6P can be oxidised to carbon dioxide without the production of metabolically useful energy (if only via oxidative phosphorylation) unless uncoupled phosphorylation is operating, when one would expect the energy of oxidation to be vented as heat. Preliminary experiments using a microcalorimeter (see Appendix C) detected no significant difference in the heat output of developing cells irrespective of myxamoebal glycogen content (Table 26) and so it is concluded that glycogen is oxidised to carbon dioxide almost certainly with the production of metabolically useful energy.

One further point is that since Wright et al (1964) reported that extracellular glucose concentrations as low as 1×10^{-4} M can affect the C6:C1 ratio, and since axenically-grown myxamoebae containing high levels of glycogen possess larger pools of glucose during

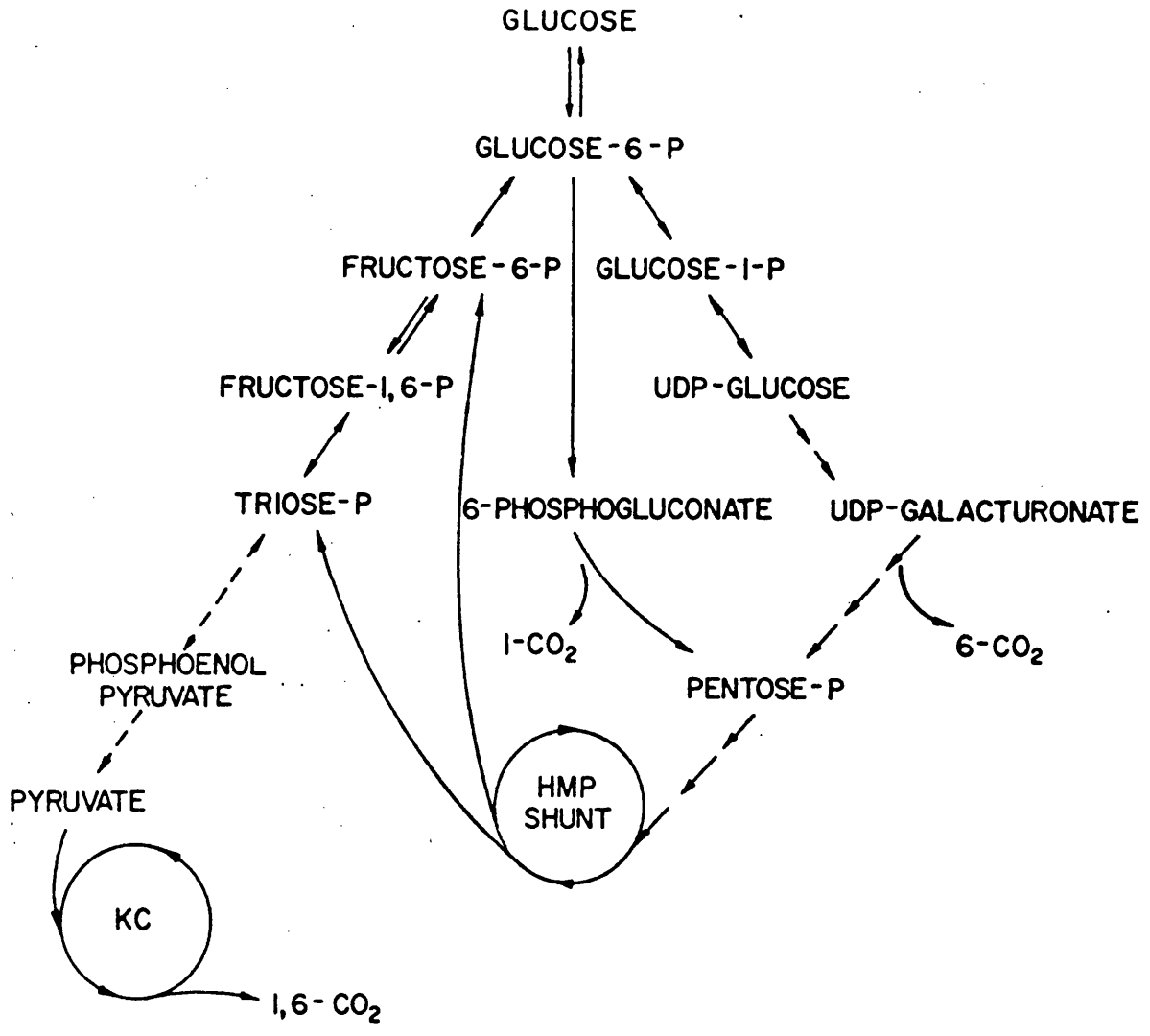


Fig.45 Schematic Representation of Possible Metabolic Pathways for Oxidation of Glucose during Development.

KC represents the Krebs cycle; HMP SHUNT represents the hexose monophosphate shunt.

(taken from Gustafson & Wright 1972)

TABLE 26 Comparison of the heat output of axenically-grown myxamoebae, initially containing various amounts of glycogen, during development

Cellular glycogen content (mg/10^8 cells)	*Heat output during 2-22 h period of development (cal./10^8 cells)	** Percentage heat output
0.29	40.64	100.00
0.31	34.73	85.60
4.51	37.71	92.80

* See Appendix C for details of calculation

** Percentage heat output = $\frac{\text{Heat output of myxamoebae during 2-22 h period of development}}{\text{Heat output of myxamoebae initially containing 0.289 mg glycogen/}10^8\text{ cells during 2-22 h period of development}} \times 100\%$

NOTE: 1 gm of glucose oxidised under standard conditions (25°C, 1 atmos. pressure) to carbon dioxide and water yields 3.74 Kcal energy. Thus the oxidation of 4.5 mg of glycogen via an uncoupled phosphorylation route should yield approx. 17 cal heat energy. In fact, (see Table 26 above) this extra heat output is not observed.

development than myxamoebae initially containing low glycogen contents (Table 27), it is possible that the metabolism of cellular glucose occurs via different routes depending on the myxamoebal glycogen content.

In summary, the evidence so far discussed shows that during the development of myxamoebae containing low levels of glycogen, protein and RNA are rapidly degraded; the protein is metabolised to ammonia and carbon dioxide presumably yielding energy, the ribose and possibly the carbon skeleton of the RNA bases may also be oxidised as energy sources. When myxamoebae possess large quantities of glycogen, they degrade it and oxidise most of the products to carbon dioxide, almost certainly producing metabolically useful energy.

We are now in a position to answer the second question; does the use of glycogen as an energy source during development inhibit or delay the use of protein and RNA? The answer is no. The data presented in this chapter show that the oxidation of as much as 5.0 mg glycogen per 10^8 cells has little effect on either the timing, rate or extent of protein degradation or on the timing, rate or extent of RNA degradation during development. Not only this but there is no accumulation of amino acids when cells oxidise large amounts of glycogen during development, indicating that the metabolism of amino acids is also unaffected. But are the amino acids still oxidised to carbon dioxide? To answer this, myxamoebae whose protein was labelled as a consequence of growth in axenic medium containing (U-^{14}C)-aspartate were allowed to develop and any radioactive carbon dioxide produced was trapped in alkali and estimated by radioactive counting (see RESULTS).

TABLE 27 Cellular glucose concentration during the development of axenically-grown myxamoebae initially containing various amounts of glycogen

Myxamoebal glycogen content ($mg/10^8$ cells)	Mean cellular glucose concentration during the 0-15 h period of development (10^{-3} M)
0.284 ± 0.024 (2)*	0.19 ± 0.03 (9)**
4.570 ± 0.490 (2)*	0.55 ± 0.11 (10)**

Results are given as means $\pm$ S.E.M.

* Figures in brackets refer to the number of experiments

** Figures in brackets refer to the number of determinations

Note: Calculation of cellular glucose concentration included the assumption that the intracellular volume of 10^9 myxamoebae is 0.5 ml.

This study showed that the oxidation of up to 2.81 mg glycogen per 10^8 cells has no significant effect on the extent of protein oxidation to carbon dioxide. In agreement with this, measurements of ammonia evolution by developing cells of D. discoideum indicate that high intracellular levels of glycogen have little effect on either the rate or extent of ammonia excretion.

Clearly, the metabolism of glycogen as an energy source during development has no effect on the degradation of protein and RNA, nor on the oxidation of the resulting degradation products.

In other microbial systems (for example, E.coli, Aspergillus) protein and RNA are readily degraded upon starvation (Dawes & Ribbons, 1964; Mizunuma, 1963, respectively). The phenomenon is believed to be triggered by the cessation of growth rather than by starvation per se (see Mandelstam, 1960 for review) since cessation of growth can apparently activate proteases and ribonucleases which normally exist in vivo in an inactive state (Harris, 1946; Berg, 1950; Elson & Tal, 1959; Chaloupka, 1960). However, whenever a metabolisable carbohydrate reserve, such as glycogen, is available intracellularly it is degraded immediately upon cessation of growth and only after exhaustion of this energy supply are protein and RNA broken down, or breakdown of the latter compounds occurs at a slower rate*(Dawes & Ribbons, 1964; Mizunuma, 1963; Strange et al, 1961). Similarly, when cells of D. discoideum are grown in axenic medium lacking added glucose, entrance into the stationary phase of growth is characterised by a rapid decay of cellular protein and cell lysis. However, if glucose is added to the medium during exponential growth, as the cells

* Certain organisms, for example, Sarcina lutea (Burleigh et al, 1963) are able to synthesise extensive reserves of carbohydrate during growth but do not degrade it during starvation, such that protein and RNA degradation are unaffected.

enter stationary phase they accumulate glycogen (Chapter I) and during stationary phase this glycogen is degraded but cellular protein is conserved and the cells do not lyse until much later (Weeks, personal communication). Yet, once these cells embark upon development, protein is degraded! Thus although myxamoebae containing high levels of glycogen have the capacity to spare protein degradation, during development they do not do so.

Apparently, protein degradation can occur in the absence of development*, but development cannot occur in the absence of protein degradation!

Axenically-grown myxamoebae also degrade RNA upon entrance to the stationary phase of growth (Leach, C.K., personal communication) but it is not known whether the metabolism of accumulated glycogen can prevent RNA degradation, although one might expect this to be so by analogy with starving bacterial systems (Dawes & Ribbons, 1964).

Obviously, important differences exist in the metabolic strategy of starving and developing cells of D. discoideum. During starvation, the prime aim of any cell must be survival. To achieve this, endogenous reserves are mobilised - the efficiency of mobilisation often determining the efficiency of survival (e.g. Harrison & Lawrence, 1963)- but it is in the interest of the starving cell to metabolise the most expendable energy sources which are not needed for cell growth, such as accumulated carbohydrate, before destroying precious proteins and RNAs essential for growth. However, whilst developing systems

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*White & Sussman (1961) have shown that protein and RNA degradation occurs in starving morphogenetically deficient mutants, and Weeks (unpublished observations) found that the development of D. discoideum strain Ax.2 can be prevented by cycloheximide at concentrations that fail to have any effect on the protein breakdown.

must also order their metabolism so as to ensure survival, certain biochemical changes must be undertaken if development is to be successful. Two of these changes seem to be that protein and RNA, even though not essential as energy sources, must be degraded. Why?

In order to achieve the differentiated state, a developing cell of D. discoideum has to synthesise many novel enzymes (see Loomis, 1970 for refs.) but once these have fulfilled their role, they are removed from the cell, either by protein degradation or excretion. This removal is an extremely specific process. Thus, during the development of axenically-grown myxamoebae, phosphorylase activity decreases rapidly during culmination whereas glycogen synthetase decays only slowly at this time (Chapter II), although both decay faster than general cell protein. UDPG pyrophosphorylase is destroyed in stalk cells but remains stable in spore cells (Ashworth & Sussman, 1967). Moreover, the decay of enzyme activity often requires RNA and protein synthesis (e.g. Sussman, M., 1965; Roth & Sussman, 1968; Loomis, 1970; Firtel &Bonner , 1972) and is therefore not a trivial event in morphogenesis. This specific removal of cellular proteins during development is not confined to enzymes which have been synthesised de novo during this phase. Thus trehalase (Ceccarini, 1967, Chapter III) and amylase (Weiner & Ashworth, 1970, Chapter II) are present in large amounts in vegetative myxamoebae but decay rapidly upon the onset of development, trehalase being excreted at aggregation and amylase activity decreasing much faster than general protein breakdown throughout the developmental phase. Other vegetative cell enzymes, for example, proteinase and ribonuclease (Weiner & Ashworth, 1970) decay only slightly faster or at

the same rate as general protein breakdown.

Clearly, specific protein removal may be as important for successful development as specific protein synthesis. Although to date little attention has been given to the detailed process of protein degradation during development, it is possible that much of it is essential not only for energy production but also to remove proteins which have served their purpose and whose presence is undesirable or even deleterious for future morphogenesis.

Furthermore, protein degradation is probably not only required to provide precursors for the synthesis of developmentally important enzymes but may also be important in the provision of precursors for end-product saccharide synthesis. In the previous chapters it has been shown that during the development of myxamoebae containing low levels of glycogen a pool of "developmental glycogen" is synthesised and that it is this, and not "myxamoebal glycogen" which is degraded at the time of end-product saccharide synthesis, possibly acting as precursor for the process. Moreover, myxamoebal glycogen cannot fulfil the role of developmental glycogen since the latter is still synthesised even when the developing cell possesses large reserves of myxamoebal glycogen. Hence, the synthesis of developmental glycogen appears to be essential to development and is known to be synthesised via gluconeogenesis, probably using the products of protein degradation as substrate. For all these reasons, protein degradation may be essential to development even in the presence of an alternative energy source.

Let us now consider the breakdown of RNA during the developmental

phase of D. discoideum. Hanks (1967) and Cocucci & Sussman (1970) have shown that the majority of RNA degraded is ribosomal RNA; vegetative cell ribosomes are replaced during the developmental phase by newly-synthesised ribosomes (Cocucci & Sussman, 1970). The question facing all who work in this field is why do the cells degrade ribosomes which functioned perfectly well during growth and synthesise others for the developmental phase, at a time when they lack any external source of nutrients. Clearly, cells which were able to use old ribosomes for development might be expected to have a selective advantage. "It might be that, during morphogenesis, only newly-made ribosomes can convey nascent mRNA to the cytoplasm and/or effect its translation. Studies of HeLa cells have indicated that mRNA transport into the cytoplasm does continue for some time after the cessation of ribosome synthesis (Darnell, 1968), but it should be noted that these cells were exponentially growing ones. In the present instance, it is necessary that transport be sustained for a period of 24 h by stationary phase cells engaged in a morphogenetic program.

A second possibility is that the ribosomes synthesized during fruiting body construction constitute a new class of ribosomes that are functionally different from ribosomes present during the cell growth and division cycle with respect to their capacity to transport and/or to translate mRNA that is transcribed during fruiting body construction. In this connection it is of interest to note that, in heterokaryons of HeLa cells and hen erythrocytes (whose cytoplasm had been allowed to leak out prior to cell fusion), the previously

quiescent hen nuclei synthesized RNA for the first few days but no RNA was found in the cytoplasm, whereas RNA newly made in the HeLa nuclei was found there; only after nucleoli developed in the hen nuclei and ribosome synthesis began did hen RNA appear in the cytoplasm and could hen-specific proteins be detected shortly thereafter (Harris et al., 1969)." (Cocucci & Sussman, 1970).

Clearly, therefore, a major reason for developmental RNA degradation could be that ribosomes present during growth are not able to function in the transport and/or translation of mRNA essential for successful development, and so are degraded, partly to provide the precursors for the synthesis of ribosomes which can carry out these functions.

Additional support for this view comes from experiments performed with levorphanol (Sussman, 1967) which inhibits ribosome synthesis. These studies indicated that the continued fabrication of new ribosomes is essential for normal slime mould development. Also, Firtel (personal communication) has recently obtained evidence that the RNA of one of the subunits of ribosomes present during the development of D. discoideum is different from that present during the growth phase [although Sussman (1967) found no difference in ribosomal RNA from growing or developing cells of Polysphondylium pallidum.]

These then are some reasons why the degradation of protein and RNA may be necessary to the developing slime mould for reasons other than as a means of producing energy. However, energy is almost certainly produced by the oxidation of protein (and possibly RNA) degradation products so that cells which oxidise large reserves of

glycogen will have more energy than is needed to sustain the developmental process. What is this "extra" energy used for? Certainly some is used for the increased synthesis of end-product saccharides which occurs in these cells, especially the massive increase in the synthesis of trehalose (the energy reserve for spore germination) (Colter & Raper, 1970). No other sink for this energy is known at present, although it does not appear to be vented as heat.

A final point of discussion concerns the trigger for development. In the more primitive systems such as sporulation in bacteria (Foster, 1956), perithecia construction in fungi (Asthana & Hawker, 1936) and fruiting body formation in myxobacteria (Dworkin,1963), differentiation is initiated at least in part by some degree of nutritional deficiency such that availability of nutrients during the early stages of differentiation can reverse the process completely. This also appears to be the case for development in D. discoideum; if cells in the early hours of development are placed in an environment containing a bacterial food supply, development ceases and growth recommences (Raper, 1956; Bonner, 1967). However, the fact that myxamoebae will develop irrespective of the cellular content of glycogen and that their glycogen is readily oxidised to carbon dioxide probably with the release of useful energy, indicates that the developmental phase of D. discoideum is not triggered by the lack of an energy source, nor by a lack of carbon. In agreement with this, Bradley, Sussman & Ennis (1956) and Krichevsky & Wright (1963) using bacterially-grown myxamoebae found that the presence of high

extracellular concentrations of a large number of amino acids, bases, glycolytic intermediates and glucose itself when tested individually failed to prevent morphogenesis.

Thus a total supply of nutrients [in the form of bacteria] can prevent development but energy and carbon sources alone cannot. Possibly, development is triggered by the cessation of growth per se, such that only the presence of all the nutrients needed for growth can inhibit the process.

Axenically-grown myxamoebae initially containing greater than 5.0 mg glycogen/10^8 cells fail to metabolise it completely during development such that mature fruiting bodies contain approximately 0.5 mg glycogen/10^8 cells, whereas myxamoebae initially containing less than about 4 mg glycogen/10^8 cells form fruiting bodies containing 0.083 ± 0.026 mg glycogen/10^8 cells (Chapter II). Thus, not only is the lack of an energy source not the trigger for development, but development can proceed and be completed in the presence of excess energy source in direct contradiction to the rather bold statement of Wright (1963) that "depletion of an exogenous energy source initiates differentiation, and depletion of an endogenous energy source may terminate the process."

The above facts force us to conclude that development is initiated and terminated by factors other than the availability of an energy and/or carbon source.

SUMMARY

(1) The metabolism of protein and RNA has been investigated during the development of *D. discoideum* using axenically-grown myxamoebae containing various amounts of glycogen.

(2) Axenically-grown myxamoebae containing less than approximately 0.32 mg glycogen/10^8 cells degrade about 70% of their protein during development to products, the majority of which were oxidised to carbon dioxide and ammonia. Whilst the TCA-soluble pool of proteins and amino acids decreased rapidly to reach basal levels during slug migration, TCA-insoluble proteins were degraded at a slower rate throughout development. Some excretion of Folin-positive material (probably proteins and amino acids) occurred during early development but ceased after 5-6 hours developmental time.

(3) During development, axenically-grown myxamoebae containing less than approximately 0.32 mg glycogen per 10^8 cells degrade about 70% of their RNA, the decay following first order kinetics. Ribose does not accumulate in the cells, nor are ribose or RNA bases excreted in large quantities.

(4) The oxidation of as much as 5 mg glycogen/10^8 cells had no effect on either the rate and extent of protein degradation or the rate and extent of RNA degradation or on the oxidation of the degradation products.

(5) The data described above together with the results of other workers was considered in terms of the interrelationship between the utilisation of protein, RNA and myxamoebal glycogen during starvation and development.

CONCLUDING REMARKS

CONCLUDING REMARKS

When conditions become unfavourable for growth, for example, when nutrients are exhausted, slime mould cells are forced to embark upon a phase of biochemical activity which will ensure their survival.

The Acrasiales are known to be capable of forming three quite distinct resistant stages - spores, microcysts and macrocysts - although not every slime mould species can produce all of these. (Blaskovics & Raper, 1957; Filosa & Chan, 1972). Whilst both microcysts and macrocysts are more resistant, for example to heat, than vegetative amoebae, they are less resistant than spores (Cotter & Raper, 1968; Blaskovics & Raper, 1957, respectively). In addition, spore formation often [although not always (Newell et al., 1969)] involves a multicellular, migratory stage which may serve in dispersal, whilst microcyst and macrocyst production lack this. One might expect therefore that spore formation would be preferred to cyst formation, and indeed, in those slime moulds studied, cyst formation appears to occur best if the conditions are both unfavourable for growth and at the same time spore production is inhibited (Toama & Raper, 1967; Blaskovics & Raper, 1957; Filosa & Chan, 1972). Dictyostelium discoideum represents an extreme case in this respect since it has not been reported to form either microcysts or macrocysts and therefore, under conditions unfavourable for growth, always embarks upon a developmental phase which terminates in spore production (Bonner, 1967). However, this is a long and complex process and so, if spores are to be successfully produced, the entire developing system must be able to

withstand potentially injurious fluctuations in the extracellular and intracellular environments.

Independence with respect to variations in the extracellular environment is fairly easy for the developing cells to achieve. Shortly after the exhaustion of nutrients, the cell sets up permeability barriers which prevent both the leakage out of the cell of valuable metabolites and interference with development by changes in extracellular conditions (Wright & Bloom, 1961; Wright, 1964).

Independence with respect to fluctuations in intracellular conditions is more difficult to attain.

The aim of the developing cells is to produce viable, resistant spores. To do this, carbohydrates must be synthesised which are not already present in the cells; cell wall polysaccharide [both to enclose the spore in a resistant cell wall and to form an erect spore-bearing stalk, the latter possibly aiding spore dispersal (White & Sussman, 1961; Ward & Wright, 1965)], mucopolysaccharide [also apparently needed for spore wall formation (Hohl & Hamamoto, 1969; Telser & Sussman, 1971)], trehalose [to serve as an energy source for spore germination (Cotter & Raper, 1970)] and glucose [both to serve as an energy source for spore germination (Cotter & Raper, 1970) and also possibly to maintain the metabolism of dormant spores (Chapter III)]. Successful development therefore necessitates that these carbohydrates (end-product saccharides) be made in sufficient amounts to ensure spore formation, survival and germination. Now, the only known substrates for end-product saccharide synthesis are UDPG and G6P and so the cell has to ensure a plentiful supply of these

metabolites at the time of end-product saccharide synthesis.

The experiments described in this thesis demonstrate that axenically-grown myxamoebae synthesise their end-product saccharides using hexose derived mainly via gluconeogenesis, probably using protein and possibly RNA as a substrate. Thus, protein and RNA, which must be degraded to provide the energy for development as well as to remove molecules which were needed for growth but may prove deleterious to development, are also used to provide the hexose for end-product saccharide synthesis. The problem is that energy is needed throughout development and so protein and RNA are degraded from the onset of this phase, but hexose for end-product saccharide synthesis is not required until the end of development. Therefore the hexose must be stored in some form which is inert until end-product saccharide synthesis begins when it can be mobilised to produce UDPG and G6P. The developing cell has apparently chosen to store the hexose as glycogen ("developmental glycogen") which may remain undegraded until a specific degradative enzyme, phosphorylase, is synthesised just before fruiting body construction.

So, protein and RNA are degraded, some of the products are used for energy production to support development and some are used to synthesise hexose which is stored until the time for end-product saccharide synthesis. In this way, the developing cell is totally independent of the carbohydrate reserves of the vegetative myxamoebae which can then be oxidised to carbon dioxide with the production of energy. Usually, if the myxamoebae have been growing on bacteria

(the source of nutrients in the wild) then the vegetative myxamoebae contain little carbohydrate reserve [approximately 50 µg glycogen/10^8 cells (Sussman & Sussman, 1969)] and so this oxidation contributes little to the total energy pool of the cell. However, when myxamoebae are grown in axenic medium containing added glucose, they accumulate large quantities of myxamoebal glycogen [>5 mg glycogen/10^8 cells.] The developing cell, as usual, oxidises this carbohydrate reserve but in so doing now produces a large amount of energy approximately equivalent to that produced from vegetative protein and RNA degradation.

In other starving systems (Dawes & Ribbons, 1964; Mizunuma, 1963) the metabolism of glycogen as an energy source delays or reduces the oxidation of protein and RNA for this purpose and this is also true for D. discoideum in the vegetative phase of growth (Chapter IV), but apparently for successful development the cell must degrade protein and RNA, probably both to remove unwanted vegetative cell material and to provide gluconeogenic substrate for developmental glycogen and hence end-product saccharide synthesis. Surprisingly, the developing cell oxidises almost all the myxamoebal glycogen and still oxidises the normal amount of protein and RNA. How it is possible for the cell to utilise glycogen as an energy source but for this energy production not to inhibit the utilisation of protein and RNA as energy sources, and, what the 'extra' energy is used for, is not understood, but uncoupled phosphorylation does not seem to be involved.

This then is one example of the versatility of the developing

system; the cell must oxidise protein and RNA during development so producing energy but can also oxidise 0.046 to greater than 5.0 mg myxamoebal glycogen/10^8 cells also with the production of energy but without interference with the processes of protein and RNA metabolism or morphogenesis in general.

High levels of myxamoebal glycogen cause one more problem to the developing cell of D. discoideum. It has only a limited amylase activity, inherited from the vegetative cell, with which to degrade myxamoebal glycogen. Thus, the amounts of myxamoebal glycogen may be so large as to prevent complete degradation before end-product saccharide synthesis begins. This results in increased cellular concentrations of glucose, UDPG, and G6P, all of which are capable of being used for end-product saccharide production. Yet one could imagine that too much end-product saccharide could be undesirable for successful development. For example, if the spore cell wall produced was too thick, this could create difficulties in the germination process which involves the uptake of water, swelling of the spore and possibly digestion of the cell wall (Cotter & Raper, 1970; Cotter et al, 1969). Apparently to prevent such disasters, the various end-product synthetic pathways are independently regulated so that the amount of cell wall polysaccharide produced is constant irrespective of the substrate pool size (although mucopolysaccharide is also part of the cell wall, it probably only forms a thin outer layer, the bulk of the wall being comprised of cell wall polysaccharide complex (Cotter et al, 1969). Thus the mucopolysaccharide may have functions, other than to strengthen the wall, which are not adversely affected by increases in the amount of mucopolysaccharide present), whilst any extra substrate is used to

make mainly trehalose and glucose. These latter two compounds are used as energy reserves during dormancy and germination (see above) and thus far from being deleterious, increased production may be advantageous.

Once more the developing cell is versatile enough to prevent potentially adverse intracellular conditions from interfering with development and even uses the adverse conditions to its advantage.

One important aspect of this versatility is that the cell must be able to respond quickly and easily to fluctuations in intracellular conditions.

Now, during the development of D. discoideum, some enzymes decrease in specific activity, others remain approximately constant, and several increase dramatically (see Chapter IV). The increase in specific activity of some of the latter has been shown to be due to de novo protein synthesis (e.g. Franke & Sussman, 1971) and there is good evidence for controls at both the transcriptional and translational levels (Sussman & Sussman, 1969). However, data from several sources indicate that the exact flux through the enzymic pathways concerned is controlled not by the cellular enzyme content [that is, not at the protein synthetic level] but rather at the metabolic level.

Thus, in Chapter III, I described experiments which demonstrate that the amount of trehalose synthesised during the development of axenically-grown myxamoebae can vary by a factor of four without any change in the cellular content of key trehalose degradative and synthetic enzymes. Control here appears to be via substrate availability.

Similarly, although the cellular content of several enzymes involved in amino acid oxidation increases considerably during development as a result of protein synthesis (Pong & Loomis, 1971), the rate of ammonia production [a measure of the rate of amino acid oxidation] remains constant throughout development (Chapter IV). Once more it appears that control of the flux through the pathway is exerted at the metabolic rather than protein synthetic level. Wright has also shown that developing cells of D. discoideum are substrate limited as regards glutamate oxidation; the cellular concentration of glutamate dehydrogenase does not change during development but the flux through the enzyme increases five-fold due to increased glutamate concentration. Weeks (in Hames, Weeks & Ashworth, 1971) has reported that the rate of glycogen synthesis during development of axenically-grown myxamoebae increases at a time when the cellular content of glycogen synthetase is actually decreasing. Finally, an alkaline phosphatase is known to increase six-fold during development but this increase in enzyme activity is cancelled out by the simultaneous accumulation of inorganic phosphate, a competitive inhibitor of the enzyme (Wright, 1966).

In all these cases it appears that the flux through the metabolic pathway concerned is controlled at the metabolic level [by variations in substrate pool size, effector concentrations etc.] and that the cellular content of enzyme is in excess of that required for the flux through the pathway.

As a general hypothesis, one might suggest that protein synthetic controls are important for development in D. discoideum in so far as they serve to ensure that the correct enzymes are present in the cell at the correct time in the life cycle, but that the enzyme produced is in excess of that required for the 'normal' (p. iv) flux through the pathway so that alterations in intracellular conditions are quickly accommodated by the existing enzymic machinery.

This system of control is not unique to the slime mould. As pointed out by Wright (1967) "substrates and effectors have been found to limit reactions necessary to both morphogenesis and catabolism in every case which has been examined" (Vetgosky & Freden, 1958; Lowry & Passonneau, 1964; Aketa et al, 1963; Krahl et al, 1955; Pannbacker, 1967; Wright et al, 1964; Wright, 1963, 1966).

If this hypothesis is correct, it should be possible for cells of D. discoideum with very different quantitative enzyme compositions to develop quite normally, provided that enough enzyme activity is present to permit essential metabolism to occur. Both Quance & Ashworth (1972) using axenically-grown myxamoebae and Sussman & Newell (1972) using bacterially-grown myxamoebae, have shown that this is so. " A given morphogenetic stage may thus contain a variety of enzyme assemblies." (Quance & Ashworth, 1972).

One point remains to be discussed. By virtue of the need for independency of variations in the external environment, the developing cell isolates itself using permeability barriers and relies on endogenous metabolism to provide both the energy and synthetic precursors for development (Wright, 1967). A large part of this

metabolism during the developmental phase of D. discoideum is concerned with myxamoebal glycogen, protein and RNA degradation and the cell possesses active catabolic enzymes for this purpose. However, successful development also requires the synthesis of specific RNA's, proteins, developmental glycogen and end-product saccharides, which may be susceptible to cellular degradative enzymes. Again the cell is faced with a dilemma; it must degrade certain cell molecules but retain others of a similar chemical nature. There is no doubt that specific mechanisms exist which are able to select which RNA's to degrade and which to conserve since some RNA's needed for developmental protein synthesis are stable for several hours during development (see Ashworth, 1971 for refs.) at a time when vegetative ribosomal RNA is rapidly destroyed (Hanks, 1967; Cocucci & Sussman, 1970). Similarly, cellular proteins are degraded at widely different rates (Chapter IV). But what about carbohydrates? How are they selected for degradation or conservation? Much evidence now suggests that developmental glycogen (Chapter II), trehalose (Chapter III), mucopolysaccharide (Hohl & Hamamoto, 1969; Telser & Sussman, 1971) and cell wall polysaccharide (Rosness, 1968) are all synthesised in compartments isolated from cellular degradative enzymes. In this way, the cell may select which carbohydrates to degrade and which to conserve.

Whilst of undoubted importance to the slime mould, compartmentalisation poses a most difficult problem for the biochemical investigator. Not only may metabolite and enzyme concentrations as measured in cell extracts bear no relation to those in isolated compartments in vivo, but there is evidence that enzymic kinetic parameters may be concentration dependent (Wuntch, Chan & Vesell, 1970). Only when the exact conditions in vivo are known will a comprehensive understanding of the control of development in biochemical terms be possible.

APPENDICES

MATERIALS

Trehalose, maltose, lactose, fructose and diethylaminoethyl (DEAE) cellulose were obtained from Sigma (London) Ltd., London W.5, U.K.; cellobiose was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.; and cellulose was obtained from Grycksbo Pappersbruk AB - Sweden.

Glycogen, purified from Dictyostelium discoideum strain Ax-2, was a kind gift of Dr. D.J. Watts.

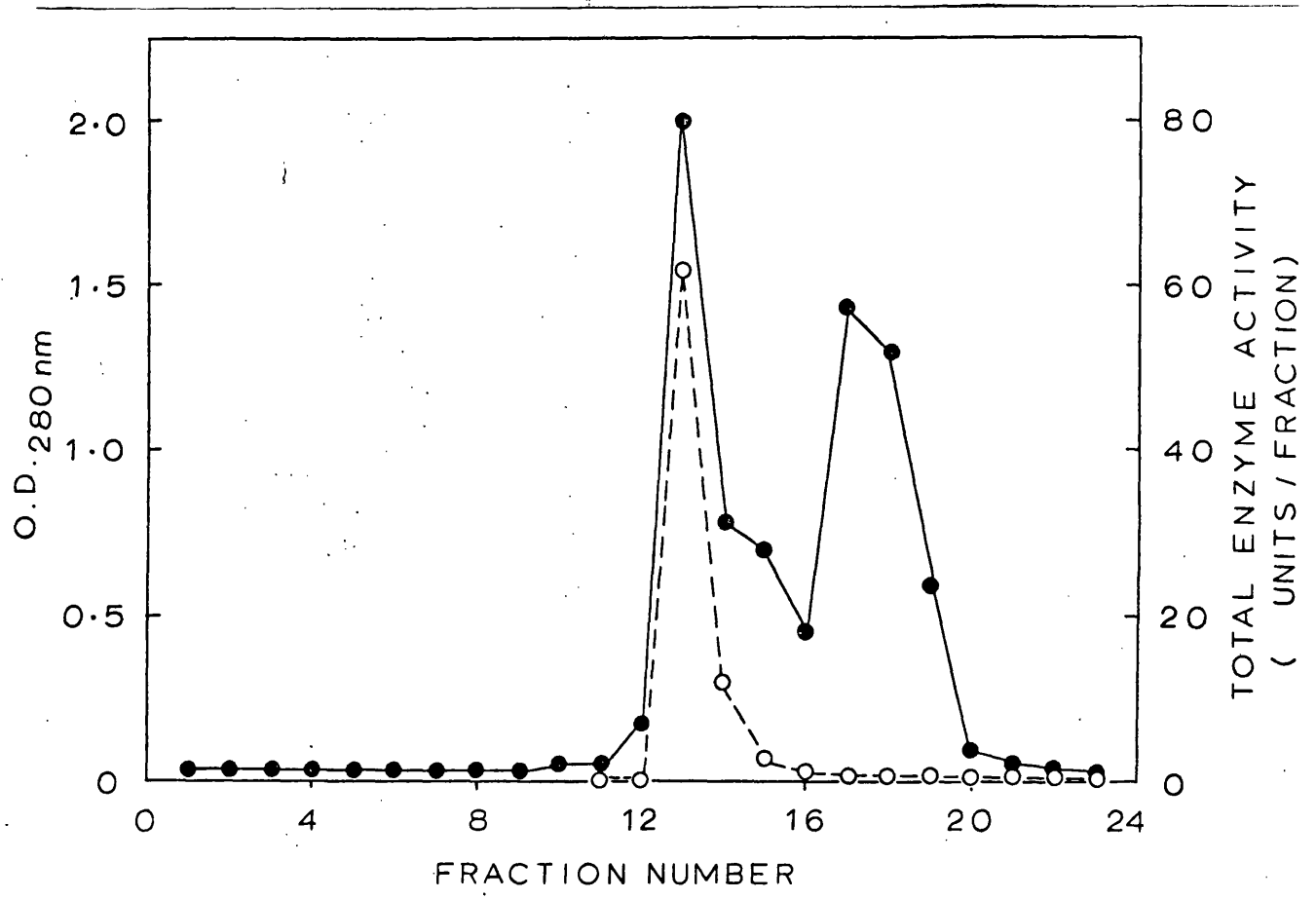
Neurospora crassa, "inositol-less" strain 89601-A, was a kind gift from The Humboldt State University Fungal Collection Centre, Arcata, U.S.A.

Appendix A

Preparation of Trehalase

Neurospora crassa was grown and harvested as described by Hill & Sussman (1963) and stored frozen at -70°C until needed. It was then mixed with two volumes of 0.05 M phosphate buffer pH 5.6 and macerated in a Waring blender. Mycelium debris was removed by centrifugation at 1000g for 10 minutes and the pellet re-extracted by grinding with sand. The resulting paste was mixed with the supernatant from the previous centrifugation and the mixture recentrifuged at 20,000g for 30 minutes. One-quarter volume of 2% ($^{w}/v$) protamine sulphate in 0.1 M phosphate buffer pH 7.0 was added to the supernatant and the mixture incubated at 0°C for 30 minutes. Following centrifugation at 20,000g for 15 minutes, the supernatant was incubated at 60°C for 15 minutes and the precipitate which formed was removed by filtration through Whatman No.4 filter paper. Ammonium sulphate was added to the filtrate to 40% saturation and after incubation overnight at 4°C, the resulting precipitate was removed by centrifugation as above. More ammonium sulphate was added to the supernatant to 80% saturation and the mixture incubated overnight at 4°C to precipitate trehalase. The precipitated enzyme was recovered by centrifugation as above, dissolved in 0.05 M phosphate buffer pH 5.6 and dialysed against this buffer at 4°C for 48 h. The dialysed enzyme was applied to the top of a column of diethylaminoethyl cellulose (DEAE cellulose), prepared according to Hill & Sussman (1963), and eluted using a gradient of 0 - 1.0 M sodium chloride in 0.05 M phosphate buffer pH 5.6 (Fig. 46). Trehalase activity was assayed as described by Ceccarini (1966).

FIG.46 Eluate Profile from First DEAE Cellulose Column.



● ; optical density of eluate at 280nm

○ ; trehalase activity of eluate (1 unit of trehalase activity is equal to the mg glucose released from trehalose by 1ml of the eluate during 30min incubation period, under the conditions of assay as described in the text).

Further purification to remove cellobiase activity was achieved by passing the trehalase preparation down a second DEAE cellulose column and using a 0 - 0.3 M NaCl gradient to elute the protein (Fig. 47).

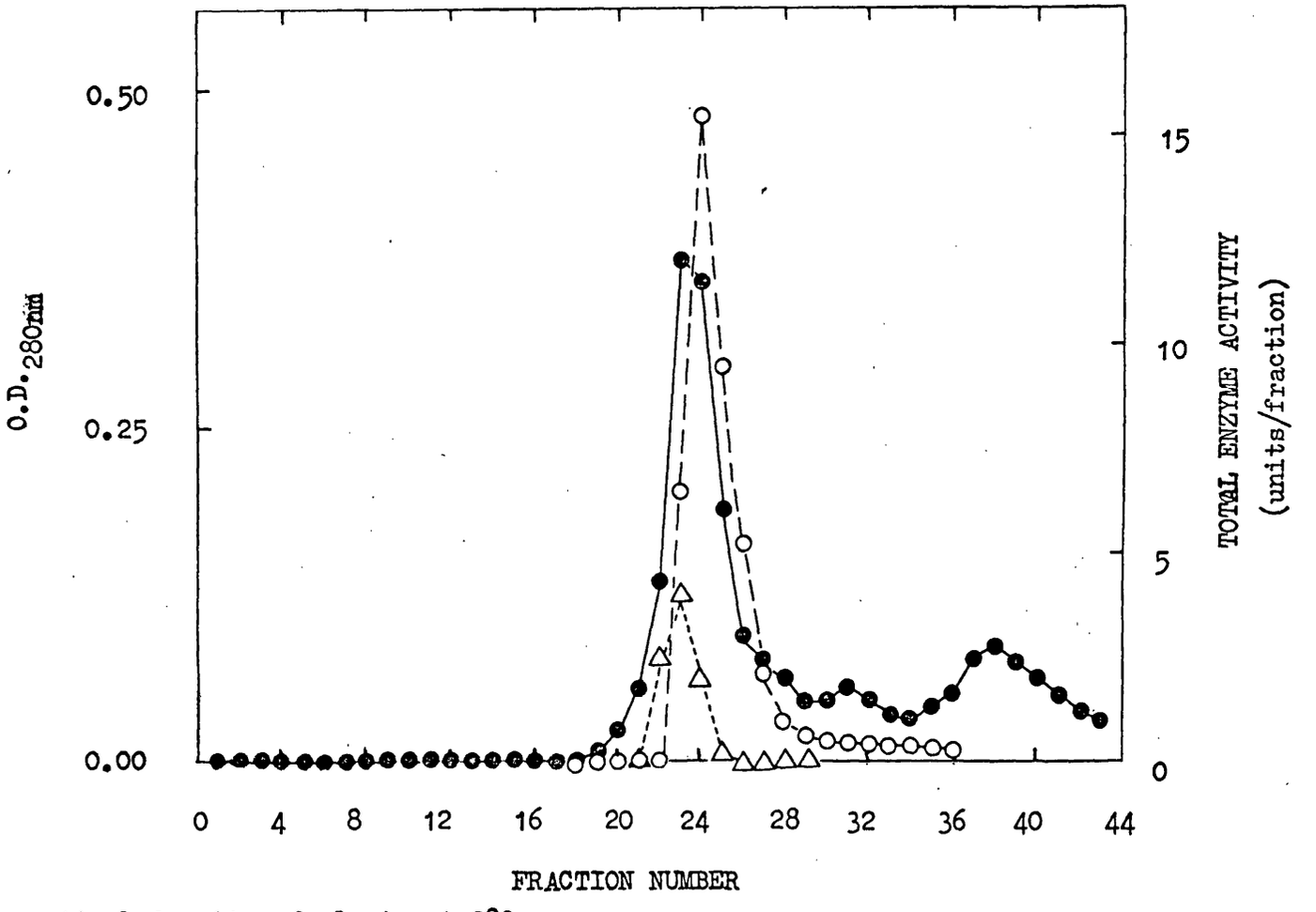
Specificity of Trehalase

The following assay was used to determine the specificity of purified trehalase.

To 0.1 ml purified enzyme was added 1.0 ml 0.05 M phosphate buffer pH 5.6 and 1 ml of the substrate to be tested [5 mg/ml in 0.05 M phosphate buffer pH 5.6]. This mixture was incubated at 37°C for 60 minutes, the reaction stopped by boiling and any glucose liberated estimated using the hexokinase assay of England & Randle (1967). Control reactions containing substrate but no trehalase were used to correct for any glucose contamination of the substrate solution.

The data presented in Table 28 indicate that the purified trehalase is specific for trehalose.

FIG. 47 Eluate profile from Second DEAE Cellulose Column.



● ; optical density of eluate at 280nm

O, Δ ; trehalase and cellobiase activities of eluate respectively (1 unit of trehalase(cellobiase) activity is equal to the mg glucose released from trehalose(cellobiose) by 1ml of the eluate during a 30min incubation period, under the conditions of assay as described in the text.

TABLE 28 The specificity of trehalase purified from Neurospora crassa

Substrate	Glucose produced (μg/h)	Percentage of Trehalase activity
* Glycogen	11.05	0.96
Maltose	0.63	0.05
Lactose	0.00	0.00
Sucrose	4.35	0.38
Fructose	0.15	0.01
Cellobiose	0.00	0.00
Cellulose	0.00	0.00
Trehalose	1150.00	100.00

* Purified from D. discoideum strain Ax-2 by D.J. Watts

Appendix B

Mass Spectrometry of the Acidic, Volatile Gas Evolved by Developing Myxamoebae of Dictyostelium discoideum

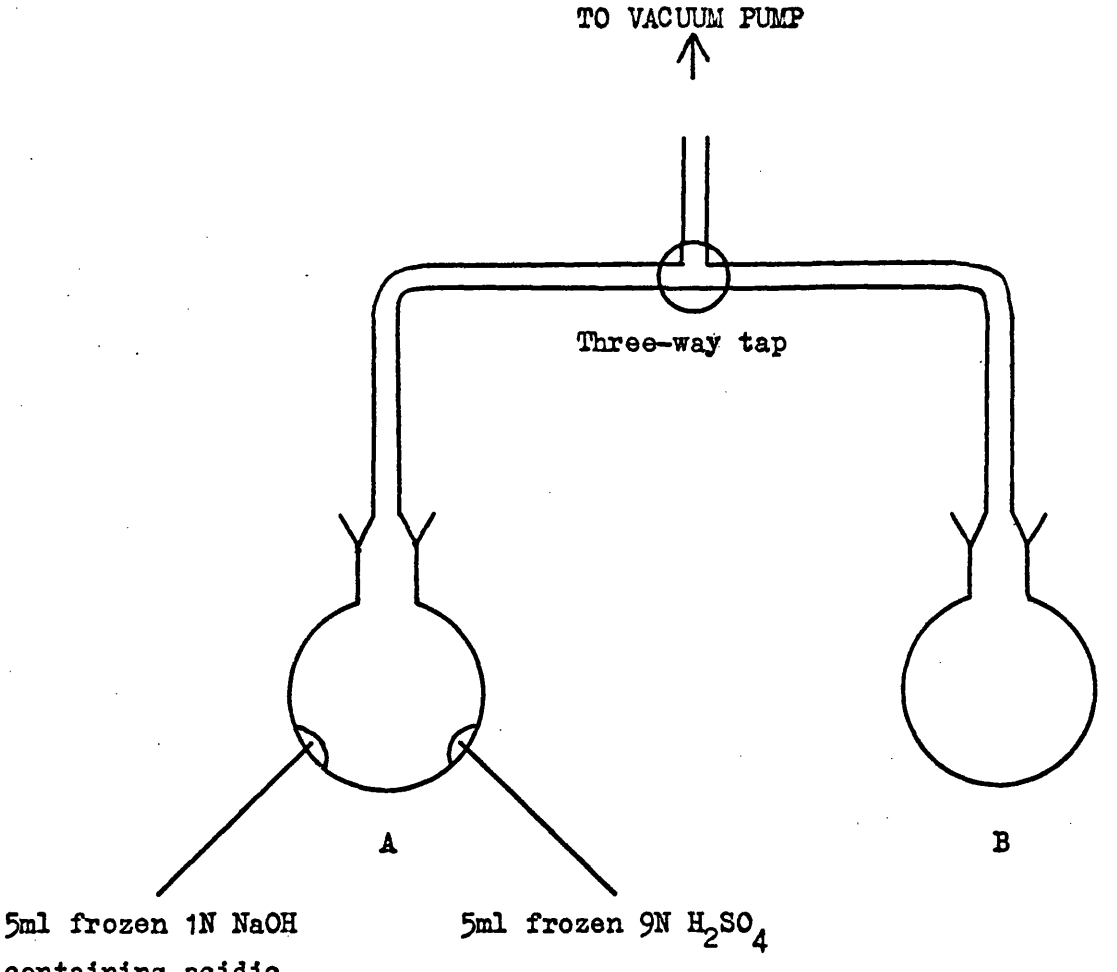
The acidic, volatile gas evolved during the development of axenically-grown myxamoebae was trapped in 1 M sodium hydroxide using the Conway dish method described in Chapter II.

A 5 ml sample of this alkali was then placed inside a 100 ml "Quickfit" conical flask and frozen using liquid nitrogen (Fig. 48). 5 ml of 9 N H_2SO_4 was also frozen inside the same flask but in a separate location from the alkali (Fig. 48). The flask [A] was now attached to the apparatus shown in Fig. 48 and the entire apparatus evacuated and then sealed using the three-way tap.

Flask B was cooled in liquid nitrogen whilst flask A was allowed to warm to room temperature [whereupon the acid and alkali melted and mixed, evolving the acidic, volatile gas] and then heated to 35°C using a water bath. After 2 hours, the three-way tap was opened to let air into flask A which was then removed and replaced with a second flask containing frozen alkali and acid pellets as before. The procedure was repeated three times in total and finally the whole apparatus was opened to the atmosphere. Flask B was quickly removed, tightly stoppered and whilst still held in liquid nitrogen, it was attached to the inlet of a mass spectrometer. Only then were the contents of flask B allowed to warm to room temperature and a spectrum determined.

The spectrum revealed that carbon dioxide was the major, perhaps only, component of the acidic, volatile gas evolved by developing myxamoebae of D. discoideum grown in axenic medium.

FIG.48 Apparatus used to trap The Acidic, Volatile Gas (evolved during The Development of Axenically-grown Myxamoebae) in an Evacuated Flask ready for Mass Spectrometry.



Appendix C

Determination of the Heat Output of Developing Cells of D. discoideum

The calorimeter used was a LKB Batch Microcalorimeter No. 10700 linked to an output recorder, and calibrated according to the following procedure.

0.30 ml water was spread over a square (sides 3cm) Millipore filter (cat. no. AABP 047 00) supported on a similar sized cellulose fibre pad (cat. no. AP 100 4700) saturated with 0.65 ml PDF. Using forceps, this Millipore filter arrangement was placed in the glass calorimeter cell, already containing 0.4 ml water, and allowed to attach itself to one wall (Fig. 49). The cell was closed with a greased, well-fitting Teflon lid and the apparatus switched on. After several hours, to allow equipment stabilisation, standard amounts of heat energy were fed to the cell by means of an electrical current and the displacement of the recorder pen noted. A standard curve of chart units displaced versus mA current was then drawn (Fig. 50).

In order to determine the heat output of developing myxamoebae, the apparatus was allowed to stabilise once more and then the Millipore filter support replaced with a similar arrangement set up as described above but using 0.30 ml of a myxamoebal suspension in water (about 5×10^7 cells/ml) instead of water. The displacement of the recorder pen over the next 30 h period (during which the myxamoebae developed) provided a measure of the heat output of the developing cells.

FIG. 49 End View of a Micro-calorimeter Cell containing a Millipore Filter Support.

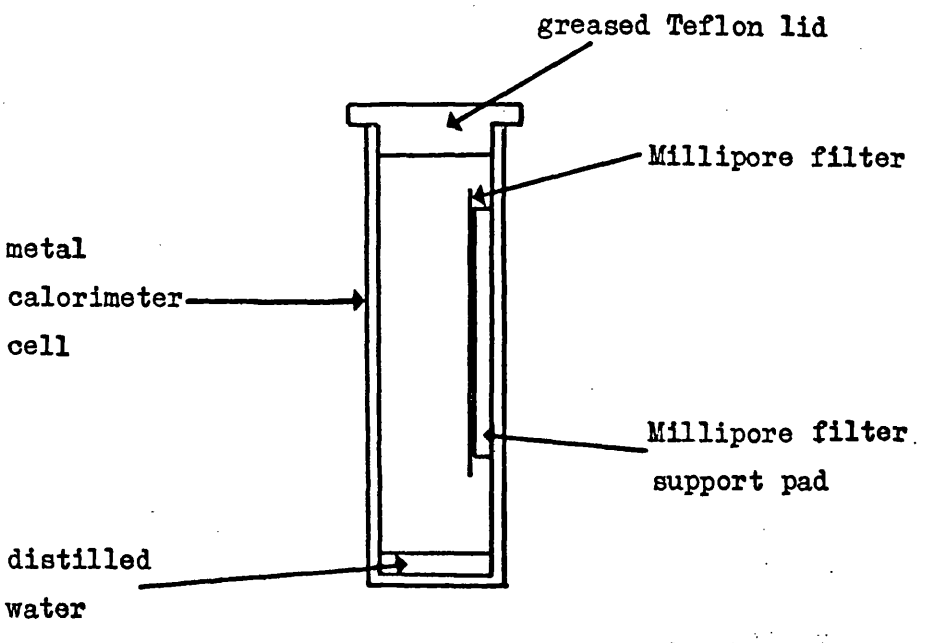
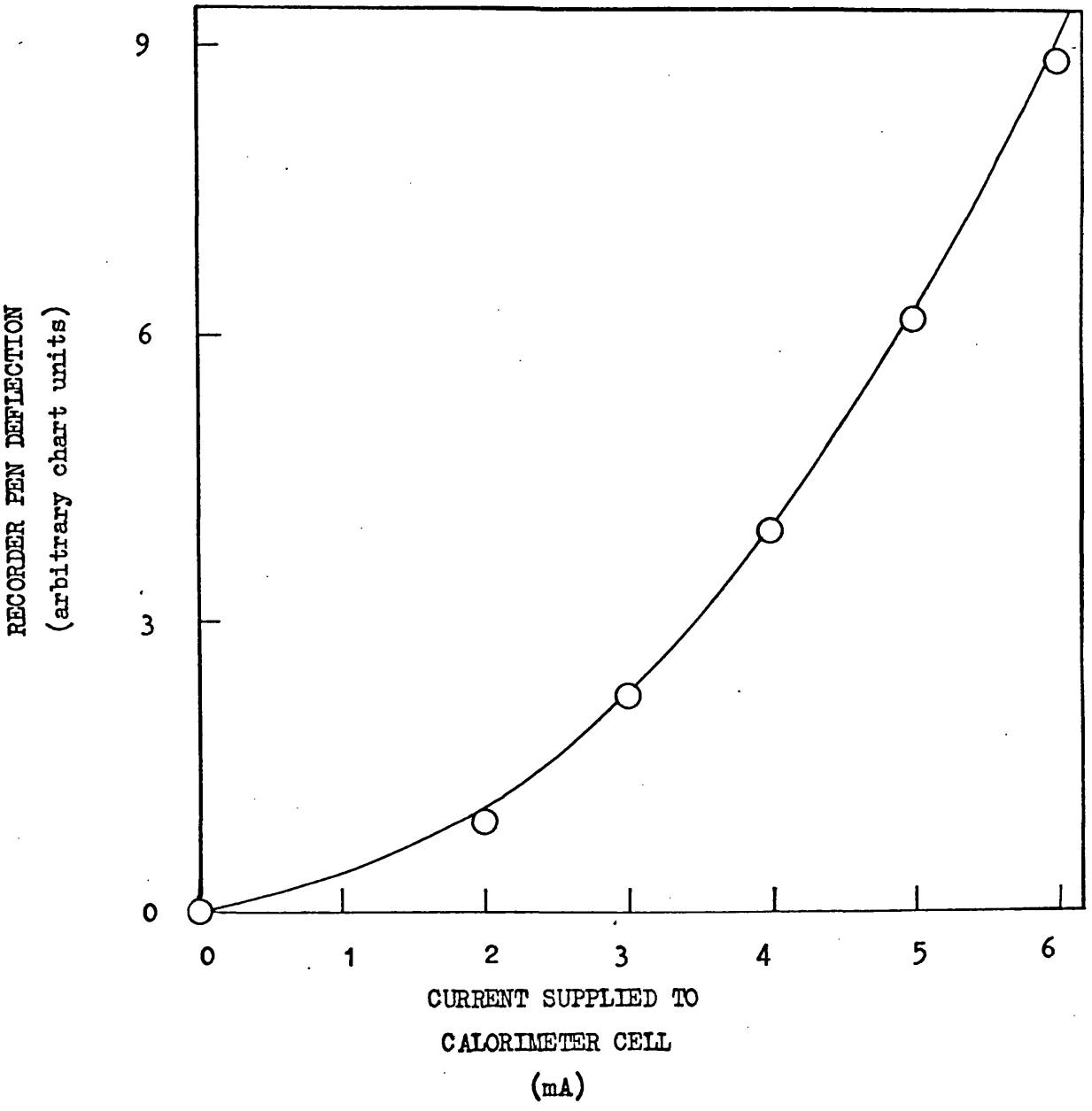


FIG. 50 Micro-calorimeter Standard Curve.



Calculation of Cellular Heat Output

The heat output of the developing cells is proportional to the area under the curve in Fig. 51.

The method of calculation is best illustrated by a worked example.

Let us suppose that cellular heat output deflected the calorimeter recorder pen 1.5 chart units for 2 hours.

Now, an equivalent deflection would be produced by passing approximately 2.5 milliampères (mA) current through the electrode in the calorimeter cell for 2 hours (h) [see standard curve Fig. 50] Thus, the area under this curve is equal to 2.5 x 2 = 5.0 mA-h.

Now,

heat energy produced $\propto I^2 R t$

where I = current
R = resistance
t = time

$\propto$ mA-h x mA x R

For this calorimeter, R = 50 ohms

and for this example, mA = 2.5

and, mA-h = 5.0

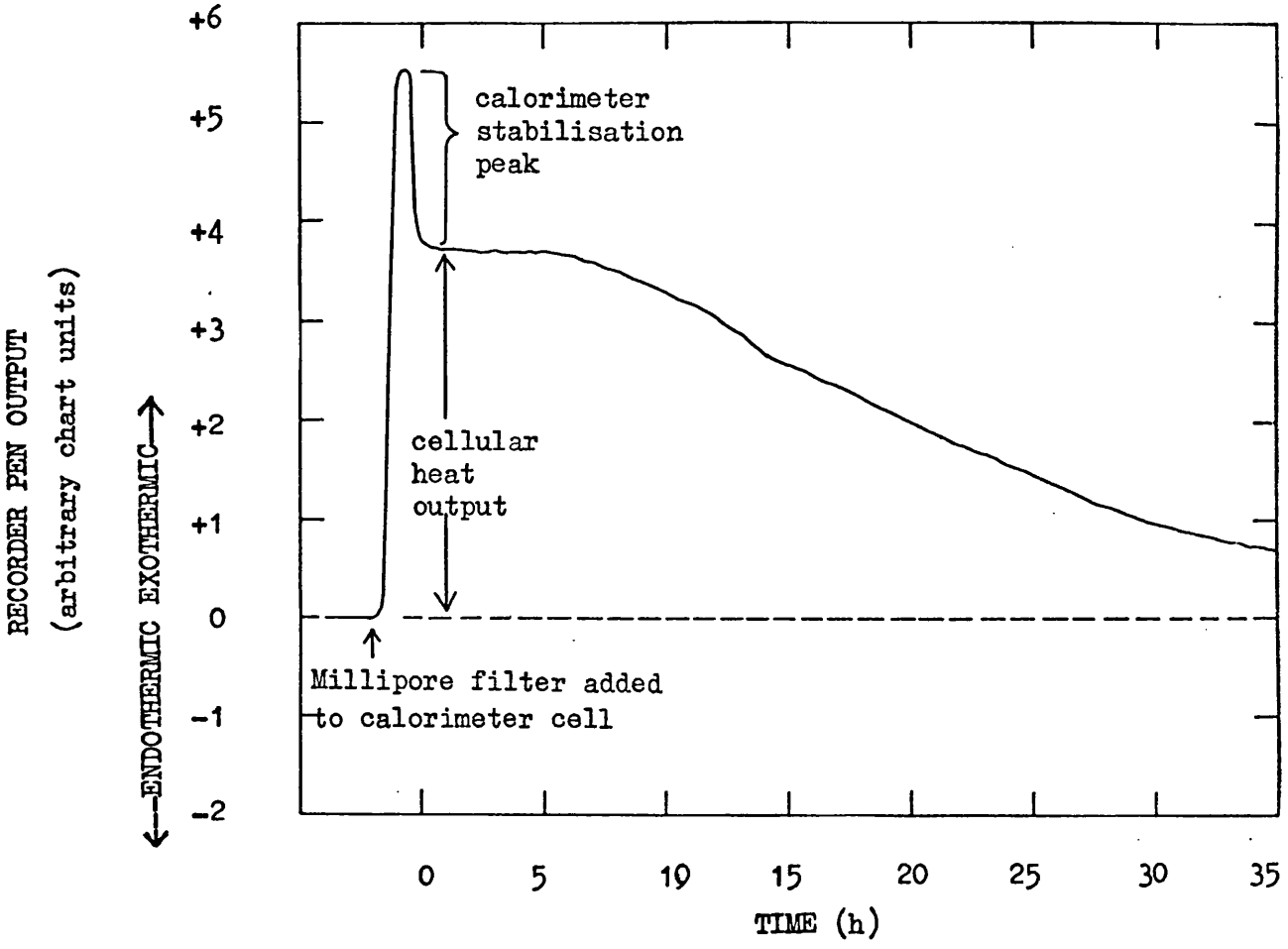
$\therefore$ Heat energy produced $\propto$ 5.0 x 2.5 x 50

$\propto$ 625 mA^2.sec.ohm

or 625 x 10^{-6} x 3600 amp^2.sec.ohm

= 2.26 amp^2.sec.ohm

FIG.51 Schematic Representation of Microcalorimeter Recorder Output During The Development of Axenically-grown Myxamoebae.



Now,

volt = amp.ohm

$\therefore$ = 2.26 amp.volt.sec.

and watt = amp.volt

$\therefore$ = 2.26 watt.sec.

and 1 watt = 0.239 cal.sec^{-1}

$\therefore$ = 2.26 x 0.239 cal.

= <u>0.54 cal</u>.

Thus the heat energy output of the cells during the 2 hour period is 0.54 cal.

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OWN PUBLICATIONS

Part of the work presented in this thesis has been previously published, the refernces being:-

Hames, B. D., Ashworth, J. M. (1971) Abstracts First International Mycological Congress; Exeter

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Glycogen Synthetase and the Control of Glycogen Synthesis in the Cellular Slime Mould *Dictyostelium discoideum* during Cell Differentiation

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1. The variation in cellular glycogen content of differentiating cells derived from myxamoebae that initially contained a wide range of glycogen contents (0.047–5.56mg of glycogen/10^8 myxamoebae) has been studied. 2. Myxamoebae that initially contained 0.047–3.62mg of glycogen/10^8 myxamoebae all gave rise to fruiting bodies that contained similar amounts of glycogen (0.06–0.11mg of glycogen/10^8 cells) but myxamoebae that initially contained 5.56mg of glycogen formed fruiting bodies containing 0.5mg of glycogen/10^8 cells. 3. Despite the high net rate of glycogen disappearance (during cell differentiation) from cells that contained more than 2mg of glycogen/10^8 cells initially, there were still significant variations in the rate of glycogen synthesis. The rate of glycogen synthesis reached a peak at the aggregation stage. 4. Evidence is presented showing that the rate of this synthesis of glycogen is controlled by factors other than the intracellular concentration of glycogen synthetase. 5. Our results are discussed in the context of the theory that the rates of glycogen synthesis and degradation act as a control mechanism for cell differentiation. 6. Criteria are discussed for deciding whether a biochemical event is causally or secondarily related to morphogenesis.

The glycogen content of the myxamoebae of the cellular slime mould *Dictyostelium discoideum* Ax-2 is a function of both the composition of the growth medium used (Ashworth & Watts, 1970) and the phase of the growth cycle (Weeks & Ashworth, 1972). Despite these changes in chemical composition, and associated changes in enzyme content (Quance & Ashworth, 1972) the myxamoebae differentiate normally to produce similar fruiting bodies by a similar time-schedule and in a similar morphogenetic sequence. Thus it is possible to examine the factors that control the cellular glycogen amounts during the differentiation of myxamoebae with either high or low glycogen contents. Wright *et al.* (1968) have suggested that the rate of both the synthesis and the degradation of glycogen alter, during cell differentiation of bacterially grown myxamoebae, in a way that can account for the observed amount of end-product saccharide (cellulose, trehalose and mucopolysaccharide) synthesis. Their model makes a number of predictions and these predictions can be tested by using myxamoebae of *D. discoideum* Ax-2 that contain different concentrations of glycogen.

Materials and Methods

Materials

Chemicals and radioisotopes were obtained from the sources described by Weeks & Ashworth (1972). Glucose oxidase and hexokinase were obtained from Sigma (London) Chemical Co. Ltd., London W.5, U.K.

Methods

Growth and differentiation of myxamoebae. Myxamoebae of strains Ax-2 and NC-4 were grown, harvested and washed as described by Weeks & Ashworth (1972). The washed myxamoebae were deposited on well-washed Millipore filter supports at cell densities of 2.5×10^7–5×10^7 myxamoebae/filter (Sussman, 1966) and allowed to differentiate at 22°C. Fruiting bodies were fully formed 24±2h after deposition of the myxamoebae.

Determination of DNA. At various times during the differentiation sequence cells were harvested into ice-cold water. Usually sufficient cells were harvested to give a final suspension of 4×10^8 cells in 8ml of water and these cell suspensions were then stored at −35°C. After being thawed, the cell suspensions were made up to 10ml with water and 5ml portions were sonicated with a 150W M.S.E. ultrasonic disintegrator (peak-to-peak amplitude of 10μm) for either four 15s periods (samples 0–16h) or for 12 15s periods (samples 16–30h) with continuous cooling in an ice–salt bath to prevent excessive heating. The DNA content of sonicated cells prepared in this fashion was then determined by a modification of the procedure of Giles & Myers (1965): cold 50%

(w/v) trichloroacetic acid (1ml) was added to 4ml of extract, and the mixture was incubated for 1h at 0°C. The resulting precipitate was centrifuged (1000*g*, 20min). The supernatant was discarded and the pellet was washed with two 2ml portions of cold 10% (w/v) trichloroacetic acid. The pellet was resuspended in 2.5ml of 5% (w/v) trichloroacetic acid and heated at 90°C for 30min to hydrolyse the DNA. The hydrolysate was centrifuged (1000*g*, 20min) and to 1.6ml of the clear supernatant was added 0.4ml of 50% (w/v) $HClO_4$, 2.0ml of 4% (w/v) diphenylamine in acetic acid and 0.2ml of aqueous acetaldehyde (1.6mg/ml). These reaction mixtures were then incubated at 30°C for at least 20h and the difference in extinction at 595 and 700nm was determined. The amount of DNA was estimated by comparison with solutions of known deoxyribose content that had been treated identically.

Determination of glycogen. Cell extracts were prepared as described above. A suspension of the extract (2ml) and 1ml of 90% (w/v) KOH were heated at 100°C for 20min. Insoluble material was removed by centrifugation at 1700*g* for 10min and extracted with 1ml of 30% (w/v) KOH at 100°C for a further 20min. After recentrifugation the two supernatants were combined and 0.2ml of saturated Na_2SO_4 and 8ml of ethanol were added. Incubation at 0°C for 10min precipitated all the glycogen, which was collected by centrifugation at 38000*g* for 15min. The pellet was dissolved in 3ml of water and the glycogen was reprecipitated by addition of 6ml of ethanol. Repetition of this precipitation procedure gave a white solid, which was finally dissolved in 2ml of 0.5M-H_2SO_4. This solution was heated at 100°C for 5h to hydrolyse the glycogen and the pH was adjusted to 5–6 by repeated extractions with equal volumes of 10% (v/v) *NN*-dioctylmethylamine in chloroform. The glucose concentration in this hydrolysate was then measured enzymically either by the glucose oxidase method of Dahlquist (1961) or by the hexokinase procedure of England & Randle (1967). Both methods gave identical results. Glycogen concentrations are expressed as glucose equivalents after hydrolysis/10^8 cells.

Determination of the rates of glycogen degradation and synthesis. Myxamoebae were grown with continuous shaking in 65ml portions of axenic medium containing 86mM-[U-^{14}C]glucose (390μCi/86mmol) in 250ml Erlenmeyer flasks. The myxamoebae were harvested at a density of 2×10^6–4×10^6 myxamoebae/ml, then they were washed in cold water and allowed to differentiate at 22°C on Millipore filters at a density of 2.5×10^7 cells/filter. At various times the cells from six such filters were harvested into 60ml of 1mM-tris buffer, pH7.4, containing 0.1M-glucose, 0.6g of NaCl/l, 0.75g of KCl/l and 0.3g of $CaCl_2$/l (Bonner, 1947; Marshall *et al.*, 1970). When necessary the developing cell aggregates were triturated before dilution to a final volume of 60ml so that the suspension consisted of clumps containing not more than five cells. The cell suspensions were then shaken in a water bath at 22°C for periods of up to 90min. Disaggregated cells that had been incubated under these conditions did not lyse and appeared normal when viewed under the microscope. Samples (2ml) of such suspensions were removed at 10min intervals and added to 1ml of 30% (w/v) KOH. Glycogen was isolated as described previously but with four cycles of alcohol precipitation before the final dissolution in 2ml of water. Half of this solution was then added to 10ml of a Triton X-100–toluene scintillation fluid (Fox, 1968) and counted for radioactivity at 62% efficiency in a Tracerlab scintillation counter. The remaining 1ml portion was hydrolysed in 0.5M-H_2SO_4 as described above and the neutralized hydrolysate was then assayed enzymically for glucose.

Determination of glycogen synthetase. The glycogen synthetase activity of cell-free extracts prepared from cells harvested (5 Millipores/3ml) in 0.1M-tris buffer, pH7.5, containing 1.5mM-EDTA and 2.5mM-dithiothreitol was determined as described by Weeks & Ashworth (1972).

Results

Glycogen content of cells during differentiation

The change in glycogen content of myxamoebae grown in axenic media containing glucose during their differentiation is shown in Fig. 1 and similar results for myxamoebae grown in axenic media without added carbohydrate are shown in Fig. 2.

The percentage recovery of cells from Millipore filters varies during the differentiation sequence. For example, it is much easier to wash off the slug stages (14–16h) by our harvesting procedures than it is to recover aggregating cells (0–7h). To correct for this inherent variability the results have been normalized to glycogen contents/10^8 cells by measuring, in the same sample, the amount of DNA and assuming (i) that the DNA content of 10^8 myxamoebae is 17.9μg and (ii) that the DNA content per cell does not change during differentiation. Both these assumptions are correct (B. D. Hames & J. M. Ashworth, unpublished work).

Myxamoebae that contained <0.1mg of glycogen/10^8 cells formed fruiting bodies containing 0.063$\pm$0.003mg of glycogen/10^8 cells (Fig. 2). As the glycogen content of the myxamoebae was raised the rate of glycogen degradation during their subsequent differentiation increased so that fruiting bodies containing 0.083$\pm$0.026mg of glycogen/10^8 cells are formed despite wide variations (1.49–3.62mg of glycogen/10^8 cells) in initial glycogen content (Fig. 1). Myxamoebae that initially contained 5.56mg of glycogen/10^8 cells (Fig. 1), however, formed fruiting bodies with

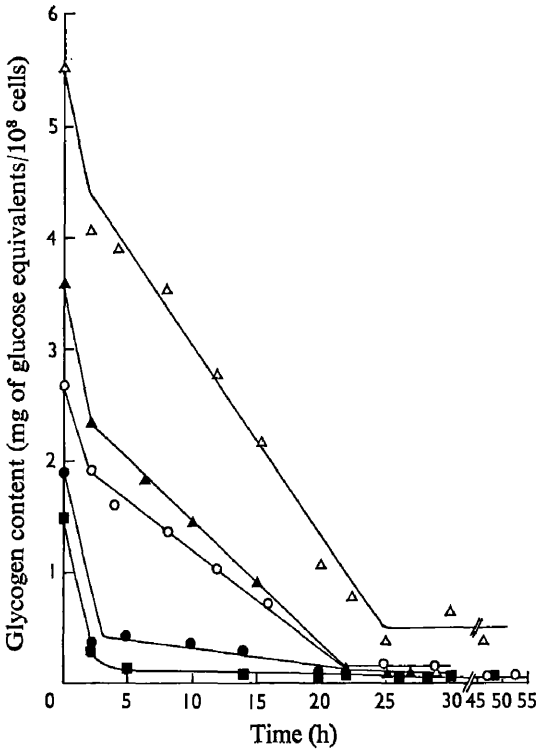

Fig. 1. *Changes in the glycogen content of cells during differentiation*

Myxamoebae were grown in axenic medium containing glucose and contained initially, per 10^8 myxamoebae: ■, 1.49mg of glycogen; ●, 1.98mg of glycogen; ○, 2.67mg of glycogen; ▲, 3.62mg of glycogen; △, 5.56mg of glycogen.

significantly more (0.5mg of glycogen/10^8 cells) than this, although the rate of glycogen degradation during differentiation of these cells was the highest we observed.

Determination of the rate of glycogen synthesis during differentiation

The decrease in glycogen content during the differentiation of myxamoebae that contained 1.49–5.56mg of glycogen/10^8 myxamoebae (Fig. 1) may be the result of two rates, one of synthesis and the other of degradation (Wright & Dahlberg, 1967). We have therefore determined the absolute rate of degradation of [^{14}C]glycogen in differentiating cells. Cells that initially contained 2.63mg of [^{14}C]glycogen/10^8 myxamoebae (see Fig. 1, points shown by ○) were allowed to differentiate for appropriate times. The cells were harvested and then incubated in the presence of [^{12}C]glucose as described in the Materials and Methods section before measurement of the radioactivity of the glycogen (Fig. 3).

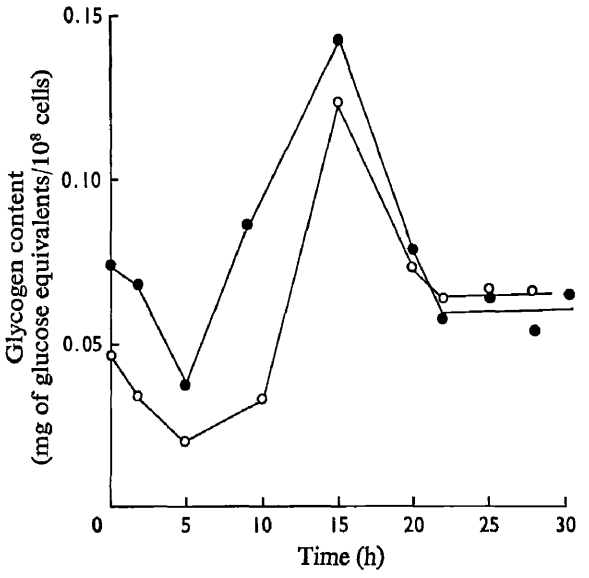

Fig. 2. *Changes in the glycogen content of cells during differentiation*

Myxamoebae were grown in axenic medium and contained initially, per 10^8 myxamoebae: ○, 0.046mg of glycogen; ●, 0.074mg of glycogen.

The absolute rate of glycogen degradation was calculated by multiplying the glycogen content and the slopes of the lines of Fig. 3. The difference between the absolute rate of glycogen degradation, so determined, and the net rate of glycogen degradation shown in Fig. 1 (○) represents the rate of glycogen synthesis. The apparent rate of glycogen synthesis increased to a maximum at 12h and then fell during the latter stages of differentiation (Fig. 4). To facilitate comparison of our results with those of Marshall *et al.* (1970), the time-period designated 'culmination' by these workers has been indicated on Fig. 4.

Glycogen synthetase activities of cells during differentiation

During the differentiation of axenically grown myxamoebae there was a continuous decrease in the activity of the glycogen synthetase, the activity at the end of differentiation being only half the activity at the onset (Fig. 5). These results were qualitatively and quantitatively different from the activities of glycogen synthetase of bacterially grown myxamoebae reported by Wright & Dahlberg (1967), who showed that the activity of the glycogen synthetase increased tenfold during aggregation, and then decreased tenfold during the remainder of the differentiation period. Therefore we also assayed the glycogen synthetase activity of bacterially grown strain Ax-2 during differentiation (Fig. 5). The change in activity of the enzyme during differentiation was qualitatively

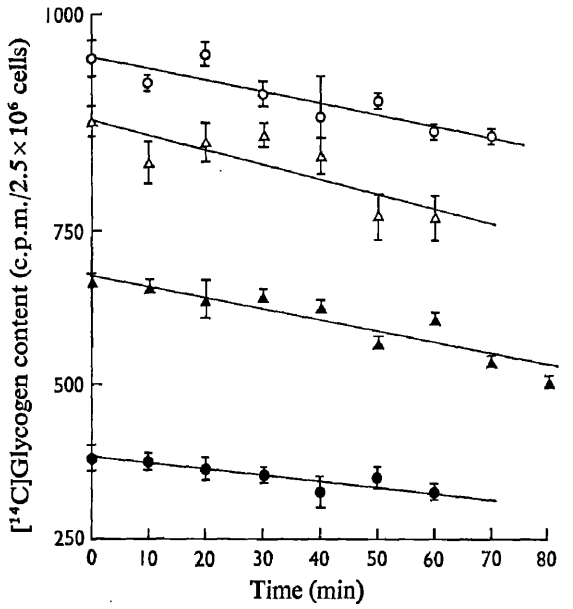

Fig. 3. *Determination of the rate of glycogen degradation during differentiation*

Myxamoebae were grown in axenic medium+86mM-[U-^{14}C]glucose and contained initially 2.6mg of [^{14}C]glycogen/10^8 cells. Cells were allowed to differentiate for ○, 8h; △, 12h; ▲, 14h; ●, 20h. They were then harvested, and the aggregates were broken up. The cells were then resuspended in a glucose–salts medium at pH7.4 and incubated at 22°C. Samples were taken at intervals and the glycogen was extracted for measurement of its radioactivity. We show representative results from three separate experiments; the 14h and 20h results come from the same experiment. Each determination represents the mean of four independent assays and the limits show the S.E.M.

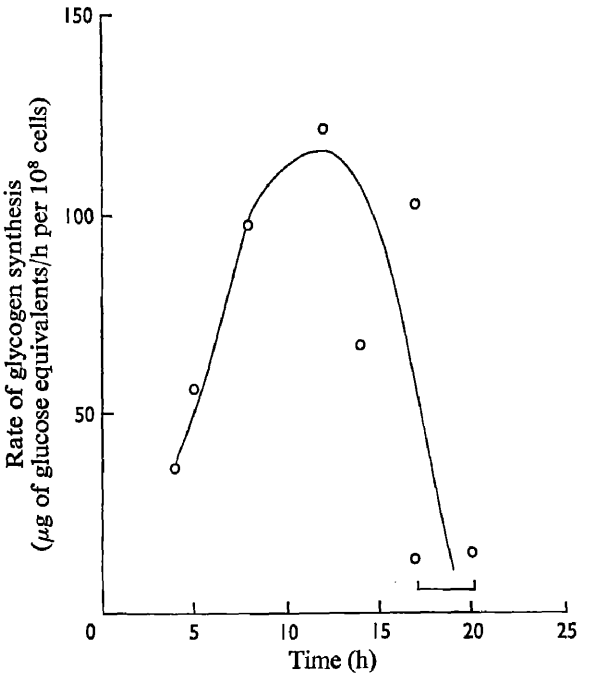

Fig. 4. *Variations in the rate of glycogen synthesis during differentiation of myxamoebae that initially contained 2.6mg of glycogen/10^8 cells*

Rates were calculated as the difference between the absolute rates of glycogen degradation (Fig. 3) and the apparent rate of glycogen degradation (○, Fig. 1). The bar denotes the 'culmination' period.

similar to that reported earlier (Wright & Dahlberg, 1967), although we only observed a twofold increase in activity at aggregation. Possible reasons for these discrepancies have been discussed in relation to the activity of this enzyme during growth (Weeks & Ashworth, 1972).

Discussion

The kinetic model of Wright *et al.* (1968) is based on the idea that 'under the steady-state conditions of the living cell and over the time-periods normally involved in the process of differentiation changes in the levels of some essential enzymes are not significant with respect to controlling the metabolite flux necessary for the accumulation of specialized end products' (Wright, 1970). The model makes a number of assumptions. One of these is that at a critical point during the differentiation an increased availability of glucose 1-phosphate (caused by an enhanced rate of glycogen breakdown) causes an enhanced rate of UDP-glucose formation (without the necessity for any increase in UDP-glucose pyrophosphorylase content) and that this in turn produces a UDP-glucose pool sufficiently increased in size to account for the observed amount of end-product saccharide synthesis. Wright *et al.* (1968) explicitly assume that: $d[\text{glucose 1-phosphate}]/dt = k_2 \cdot [\text{soluble glycogen}] - V_{UDPG}$ where k_2 is a constant and V_{UDPG} is the rate of UDP-glucose synthesis. Their model predicts that to achieve this increased availability of glucose 1-phosphate there should be a threefold increase in the rate of both degradation and synthesis of glycogen at this critical time. Marshall *et al.* (1970) have reported that this change is found during the culmination stage of the differentiation of bacterially grown myxamoebae. Our experiments were designed to test some of the assumptions and predictions of this model.

Although there was a net decrease during differentiation in the glycogen of myxamoebae that initially contained 1.49–5.56mg of glycogen/10^8 cells (Fig. 1) there was considerable glycogen synthesis, and the rate of this synthesis increased to a maximum

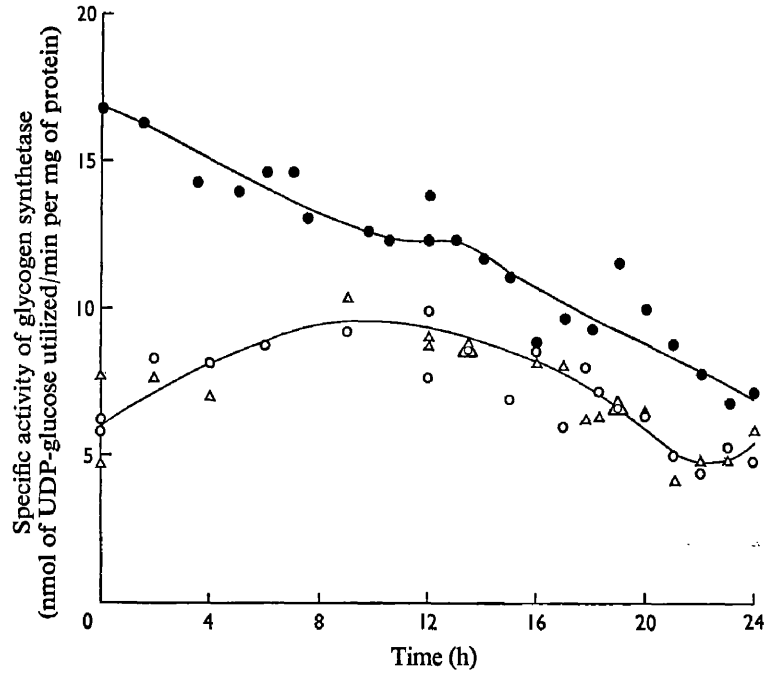

Fig. 5. *Glycogen synthetase activity of strains NC-4 and Ax-2 during differentiation*

Strain NC-4 and Ax-2 were allowed to differentiate at a cell density of 7×10^7 and 4×10^7 cells/filter respectively. At the indicated times, 2×10^8 cells were washed off the filters into 3 ml of 0.1 M-tris–chloride (pH 7.5)–0.0015 M-EDTA–0.0025 M-dithiothreitol. Suspensions were stored at −65°C until the next day and then the cells were disrupted by controlled sonication as described in the Materials and Methods section. The glycogen synthetase activity of the 5000*g* supernatant fraction was determined by measuring glycogen-dependent UDP formation for strain Ax-2 (●) and for strain NC-4 (○) and by [U-^{14}C]glucose incorporation into glycogen (△), as described in the Materials and Methods section.

at 12h. However, the specific activity of glycogen synthetase was not increasing but decreasing during this time (Fig. 5). Further, there was at all times apparently an excess of enzyme capacity. Thus at 12h the rate of glycogen synthesis observed was still only one-tenth that possible if the enzyme were operating under optimum conditions. Hence, there was no evidence to suggest that during differentiation, as during growth (Weeks & Ashworth, 1972) the rate of glycogen synthesis was controlled by the cellular content of the enzyme glycogen synthetase. This is consistent with the contention that 'changes in the levels of certain essential enzymes are not significant with respect to controlling the metabolite flux, necessary for the accumulation of specialized end products' (Wright, 1970).

However, not all our results were consistent with the kinetic model. During differentiation there could be a considerable fluctuation in the glycogen content of the cells, and comparison of Figs. 1 and 2 shows that the same morphogenetic stage (e.g. 12h) could be formed from cells performing net glycogenolysis or glycogen synthesis or with a constant net glycogen content. The kinetic model of Wright *et al.* (1968) demands that the glycogen content of the cells during the early stages of differentiation should remain constant and then decrease during culmination. Wright (1967) even suggested that the timing and spatial organization of developmental events might be determined by the utilization of a specific amount of reserve material, the rate of removal of this material being the controlling factor for the whole of morphogenesis. Clearly, such a view is difficult to reconcile with the results shown in Fig. 1. The considerable variation possible in the glycogen content of differentiating cells is much more consistent with the results of White & Sussman (1961), who found that the pattern of changes in the glycogen content of bacterially grown cells was qualitatively very similar to that shown in Fig. 2. However, whereas they suggested that the initial decrease was caused by the final utilization of ingested bacterial glycogen our results with axenically grown myxamoebae imply that this decrease is likely to be a genuine developmentally controlled event.

Another of the assumptions of the kinetic model is that there is no gluconeogenesis during differentiation, and Cleland & Coe (1969) showed that the

amount of gluconeogenesis during the differentiation of bacterially grown myxamoebae was slight. However, our results suggest that during differentiation there can be a significant increase in the glycogen content of the cells that initially have a low glycogen content (Fig. 2). This increase is caused by gluconeogenesis, because there is a concomitant increase in the total anthrone-positive material during the first 15h (B. D. Hames & J. M. Ashworth, unpublished work).

Finally, the kinetic model requires that the rates of glycogen synthesis and degradation increase between aggregation and culmination and results that support this prediction have been reported (Marshall *et al.*, 1970). We have determined the rate of glycogen degradation in cells containing a relatively high initial glycogen content by a procedure identical with that of Marshall *et al.* (1970). This procedure involves resuspending developing cells in a glucose–salts medium and measuring the rate of the ensuing loss of radioactivity from the glycogen. We assume that the measured degradation rate is similar to that of the developing cells although, because the cells cease to differentiate when washed off the Millipore filters, this may not be strictly correct. Unfortunately, it is impossible to perform experiments of this kind with cells on the Millipore filters, since we cannot ensure that all of such cells are exposed to the same concentration of non-radioactive glucose. However, it is unlikely that any gross change occurs in the cells during the relatively short periods of incubation (90min), as the cells recover and continue to differentiate if replaced on Millipore filters. Further, we are interested in changes in rates of degradation rather than in the absolute values, and it is probable that any metabolic changes introduced by this technique will be the same with different samples of cells. We find that cells with a relatively high initial glycogen content have a maximum rate of glycogen synthesis at aggregation (Fig. 4). This is in contrast with the results of Marshall *et al.* (1970) who found that in myxamoebae grown on bacteria the maximum rate was at culmination. Interestingly, the variation in the rate of glycogen synthesis during differentiation of cells with a high initial glycogen content (Fig. 4) parallels the variation in glycogen content of cells with a low initial content (Fig. 2). Thus it is possible that the net decrease in glycogen content of cells with high initial glycogen content masks the synthesis of a specific pool of glycogen that is of developmental significance.

The present paper and those preceding it (Edmundson & Ashworth, 1972; Ashworth & Quance, 1972; Quance & Ashworth, 1972; Weeks & Ashworth, 1972) are concerned with the nature of the biochemical events characteristic of cell differentiation in *D. discoideum*. In principle it might be expected that there would be two classes of such events. One class would consist of those that are necessarily involved with the characteristic progressive changes in morphology that define the differentiation of the cells. The other class of biochemical events would then consist of those necessary to maintain the vital functions of the cells despite the changing environment and interactions imposed on the cells by the morphogenesis of which they are part. It is difficult to distinguish between these two classes of events. It is, however, important to make such a distinction, because only events of the first class can be said to 'cause' cell differentiation; events of the second class are merely secondary effects. However, because the initial physiological state of the differentiating myxamoebal cells can be altered by altering the conditions under which they are grown without thereby affecting the morphogenesis it is possible to distinguish between these two classes of event. If a presumptive developmental event alters during the differentiation of different myxamoebal populations then this event must be considered to be a class two event as defined above. If such an event does not alter then we here consider it to be a class one event. However, logically this criterion, although necessary, is not a sufficient proof that the event is of the first class, because there may be an infinite number of initial physiological states and, clearly, not all can be examined. We would like to restrict the term 'developmental programme' to those events that fall into class one, as defined above.

There is no reason to expect that the distinction we have made between classes of biochemical events corresponds necessarily to different levels of control (i.e. controls of the synthesis or of the activity of enzymes). Thus whereas the synthesis of, for example, *N*-acetylglucosaminidase cannot be said to be part of the 'developmental programme' its excretion may be (Quance & Ashworth, 1972). Similarly, the synthesis of alkaline phosphatase (Quance & Ashworth, 1972) by these same criteria may be regarded as part of the 'developmental programme'. The idea that the 'developmental programme' is controlled by the rates of glycogen synthesis and degradation, and hence the steady-state concentrations of glucose 1-phosphate and UDP-glucose, seems untenable. However, although the synthesis of UDP-glucose pyrophosphorylase (Edmundson & Ashworth, 1972; Ashworth & Watts, 1970) seems to be a class one event, it is clear that the initial enzyme content of the myxamoebae is itself sufficient to account for the rate of UDP-glucose production necessary for end-product saccharide synthesis. Part of the 'developmental programme' must therefore involve the requirement that a vast excess of this enzyme must always be present. Newell & Sussman (1970) have come to a similar conclusion on the basis of very different experimental evidence.

The biochemical problem posed by the cell

differentiation of *D. discoideum* thus becomes one of studying the interactions between different levels of control of what we have termed 'class one' events.

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Metabolic Control of Development in the Cellular Slime Mould, Dictyostelium discoideum

Abstract of a thesis presented in part fulfilment of the requirements for the degree of Doctor of Philosophy of the University of Leicester.

by B.D. Hames (B.Sc.)

Axenically-grown myxamoebae of Dictyostelium discoideum strain Ax-2, containing various amounts of glycogen, develop in a manner chronologically and morphologically similar to myxamoebae of Dictyostelium discoideum strain NC-4 grown on Aerobacter aerogenes or Escherichia coli.

Isotopic and other studies indicated the presence of two incompletely separate pools of cellular glycogen during this development:-

(1) Myxamoebal glycogen; present in the cell cytoplasm in granular form unbounded by membranes and degraded immediately upon the onset of development, probably by combined amylase and maltase action, to products which are mainly oxidised to carbon dioxide.

(2) Developmental glycogen; synthesised in an unknown cellular location during aggregation, regardless of

myxamoebal glycogen content, using hexose derived at least in part via gluconeogenesis. Net degradation of this glycogen occurred only during sorocarp construction, possibly by the action of glycogen phosphorylase which reaches peak specific activity at this time.

This data is inconsistent with the model of Wright et al (1968) and was discussed in this context.

Whilst end-product saccharides (trehalose, a mucopolysaccharide, cell wall polysaccharide) may in part be synthesised from developmental glycogen, analyses revealed that myxamoebal glycogen can also act as precursor. Investigations of this system showed that the pathways of end-product saccharide synthesis are not coordinately controlled. Moreover, trehalose accumulation is regulated not by the cellular content of trehalose-6-phosphate synthase nor trehalase but rather by the cellular concentrations of UDP-glucose and glucose-6-phosphate, the substrates for trehalose synthesis.

Although some myxamoebal glycogen can be used for end-product saccharide synthesis, most was oxidised to carbon dioxide probably with the production of metabolically useful energy, but this failed to inhibit developmental protein or RNA degradation and oxidation. It is therefore suggested that protein and RNA degradation is essential for successful development for reasons other than energy production.

The remarkable versatility of metabolism during the developmental phase of Dictyostelium discoideum is discussed.