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Enzyme Synthesis in Myxamoebae of the Cellular Slime Mould *Dictyostelium discoideum* during Growth in Axenic Culture

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1. The specific activities of β-*N*-acetylglucosaminidase, acid phosphatase, α-mannosidase, β-glucosidase, UDP-glucose pyrophosphorylase and alkaline phosphatase have been determined in myxamoebae of the cellular slime mould *Dictyostelium discoideum* Ax-2 grown on different media and in different phases of the growth cycle. 2. Variations in enzymic composition occur with changes in growth medium and phase of the growth cycle. 3. The intracellular location of the enzymes studied has been determined. 4. Two enzymes, β-*N*-acetylglucosaminidase and α-mannosidase, are not only synthesized preferentially as the myxamoebae enter the stationary phase of growth but they are also excreted. The excretion process appears to be specific, because other enzymes that occur in the same intracellular fraction are not excreted.

Watts & Ashworth (1970) described the isolation of a mutant of the cellular slime mould *Dictyostelium discoideum* the myxamoebae of which are capable of growth both in a relatively simple axenic medium and with bacteria as a source of nutrients. This mutant, *D. discoideum* Ax-2, will grow in a variety of different axenic media, and the size, chemical composition and metabolic behaviour of the myxamoebae change with alterations in the growth medium (Ashworth & Watts, 1970).

The present paper describes some changes in enzymic composition that occur with changes in the growth medium and during the transition from the logarithmic to the stationary phase of growth. In particular, we have studied those enzymes that are considered to be part of the 'developmental programme' (Newell & Sussman, 1970; Loomis, 1970). The activity of these enzymes during the cell differentiation processes characteristic of this organism are discussed in the following paper (Quance & Ashworth, 1972).

Materials and Methods

Materials

p-Nitrophenyl β-*N*-acetylglucosaminide was prepared by the method of Lowry & Conchie (1966), or purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., who also supplied *p*-nitrophenyl β-glucoside and *p*-nitrophenyl phosphate. Phosphoglucomutase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. UDP-glucose and $NADP^+$ were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. All other chemicals were of the highest purity available and were the products of either BDH Chemicals Ltd., Poole, Dorset, U.K., or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Growth of D. discoideum Ax-2

Cultures of myxamoebae (700ml) were grown axenically as described by Watts & Ashworth (1970) in 2-litre Erlenmeyer flasks. Samples of the cultures were plated clonally on *Aerobacter aerogenes* periodically to check that all the cells were genetically capable of forming fruiting bodies. Myxamoebae were also grown in association with *A. aerogenes* on agar plates as described by Sussman (1966).

Assay of enzyme activities

Suspensions of myxamoebae in water were treated with Triton X-100 (final concentration 0.1%, w/v), and enzymes were assayed as follows: β-*N*-acetylglucosaminidase (Loomis, 1969*a*); α-mannosidase (Loomis, 1970); alkaline phosphatase (Loomis, 1969*c*); β-glucosidase (Coston & Loomis, 1969); acid phosphatase (Wiener & Ashworth, 1970). All these assays depend on the estimation of *p*-nitrophenol in alkaline solution by measuring E_{410}. In this paper we have used a value of 17500 for ϵ_{410} of *p*-nitrophenol. In the paper by Wiener & Ashworth (1970) an inappropriate value was used and thus the values we now report for β-*N*-acetylglucosaminidase differ from those previously reported. UDP-glucose pyrophosphorylase was assayed by method 3 of Edmundson & Ashworth (1972) with cell extracts

prepared by exposing suspensions of myxamoebae in 0.1 M-tricine [*N*-tris(hydroxymethyl)glycine] buffer, pH 8.6, to the maximum output of an M.S.E. ultrasonic disintegrator for 1 min at 0°C. Specific activities are expressed in milliunits/mg of protein, where 1 unit catalyses the formation of 1 μmol of product/min. The protein content of cell extracts was determined by the method of Lowry *et al.* (1951).

Table 1. *Effect of growth conditions on enzyme activities*

Results are expressed as means ± S.E.M. with the number of determinations in parentheses. Myxamoebae harvested at a cell density of 1×10^6–6×10^6 myxamoebae/ml in axenic medium containing glucose or at 0.5×10^6–2×10^6 myxamoebae/ml when in axenic medium alone are considered to be in the exponential (log) phase of growth and when at a cell density of $>1\times10^7$ myxamoebae/ml in axenic medium containing glucose or $>4\times10^6$ myxamoebae/ in axenic medium are considered to be in the stationary (stat) phase of growth.

Enzyme	Growth phase	Growth in axenic medium: Alone	Growth in axenic medium: With 86 mM-glucose	Growth on bacteria
N-Acetylglucosaminidase	Log	191 ± 12 (12)	204 ± 15 (16)	68 ± 7 (8)
	Stat	216 ± 26 (13)	390 ± 30 (15)	
α-Mannosidase	Log	18.9 ± 1.9 (11)	21.2 ± 1.9 (13)	2.0 ± 0.7 (4)
	Stat	9.7 ± 1.0 (13)	25.0 ± 3.5 (13)	
β-Glucosidase-1	Log	1.9 ± 0.4 (10)	4.5 ± 0.6 (10)	18.7 ± 2.6 (4)
	Stat	1.3 ± 0.2 (13)	5.0 ± 0.7 (11)	
Alkaline phosphatase	Log	19.4 ± 2.6 (6)	21.8 ± 3.5 (7)	17.3 ± 1.5 (5)
	Stat	10.4 ± 1.2 (9)	16.4 ± 1.7 (6)	
UDP-glucose pyrophosphorylase	Log	34.9 ± 2.9 (3)	41.8 ± 3.6 (4)	30.1 ± 3.1 (3)
	Stat	47.1 ± 2.8 (4)	48.8 ± 2.4 (3)	

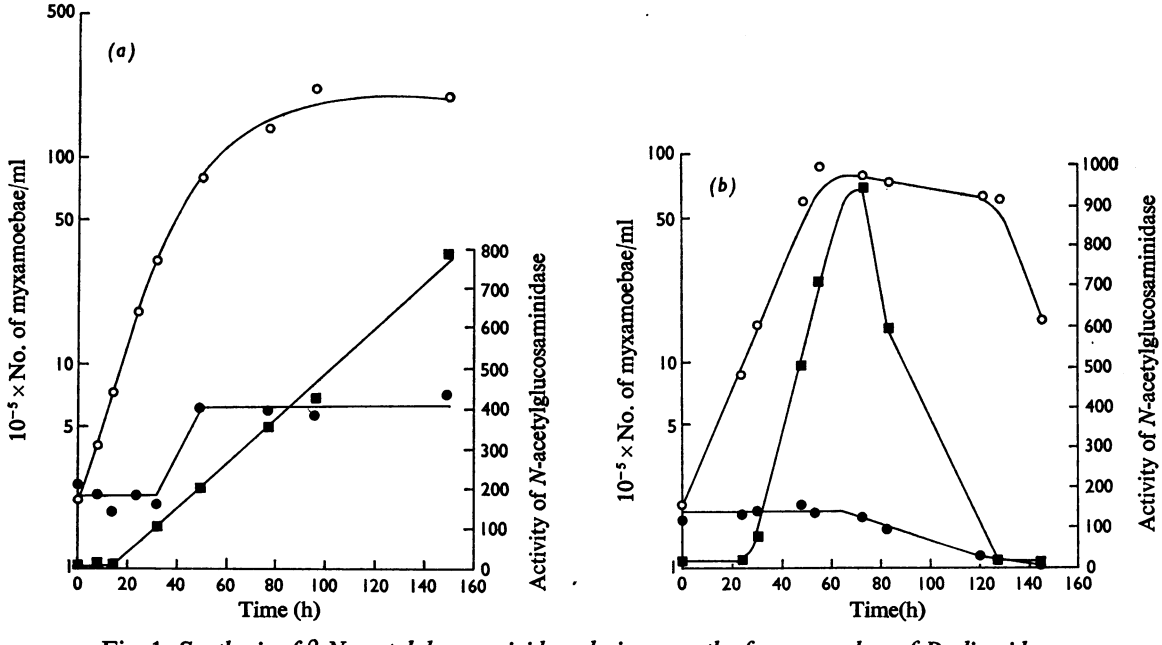

Fig. 1. *Synthesis of β-N-acetylglucosaminidase during growth of myxamoebae of D. discoideum*

The myxamoebae were grown (*a*) on axenic medium+glucose and (*b*) on axenic medium without added carbohydrate. ○, Cell number; ●, specific activity of β-*N*-acetylglucosaminidase in the myxamoebae (nmol/min per mg of protein); ■, β-*N*-acetylglucosaminidase content of the medium (nmol/min per ml of medium).

Cell fractionation

Freshly harvested myxamoebae were suspended in 0.25M-sucrose and homogenized in a Teflon–glass homogenizer until 80–90% of the cells were broken. Unbroken cells were removed by centrifugation at 400*g* for 5min and the supernatant was further fractionated by centrifugation at 15000*g* for 15min in an M.S.E. 18 centrifuge. The pellet, which contained lysosomes, peroxisomes and mitochondria (Wiener & Ashworth, 1970), is termed the 'lysosomal' fraction. The supernatant was further fractionated by centrifugation at 100000*g* for 1h to give a pellet (microsomal fraction) and the supernatant (soluble fraction).

Results

Enzyme synthesis and excretion during the growth of myxamoebae

We have studied five enzymes that have been considered to form part of the 'developmental programme': β-*N*-acetylglucosaminidase (Loomis, 1969*a*), α-mannosidase (Loomis, 1970), the β-glucosidase isoenzyme β-glucosidase-1 (Coston & Loomis, 1969), UDP-glucose pyrophosphorylase (Ashworth & Sussman, 1967) and alkaline phosphatase (Loomis, 1969*c*). Table 1 summarizes the specific activities of these enzymes in myxamoebae of strain Ax-2 grown under various conditions. The observed differences prompted an investigation of the variation in specific activity of these enzymes throughout the growth cycle. The specific activities of *N*-acetylglucosaminidase and α-mannosidase in myxamoebae growing axenically were measured (Figs. 1 and 2). Increasing amounts of these enzymes also appeared in the growth medium (Figs. 1 and 2). As high cell densities were approached in glucose-containing media (Figs. 1*a*, 2*a*) there was a doubling of the specific activity of both enzymes. The cell density at which this increase occurred was, however, different with the two enzymes. The increased synthesis of α-mannosidase preceded that of *N*-acetylglucosaminidase.

Changes in the specific activity of the other enzymes mentioned in Table 1 were also observed during the growth cycle (Figs. 3 and 4). The presence or absence of glucose in the medium had a marked effect on acid phosphatase synthesis but little or no effect on β-glucosidase synthesis or alkaline phosphatase degradation. β-Glucosidase and, to a lesser

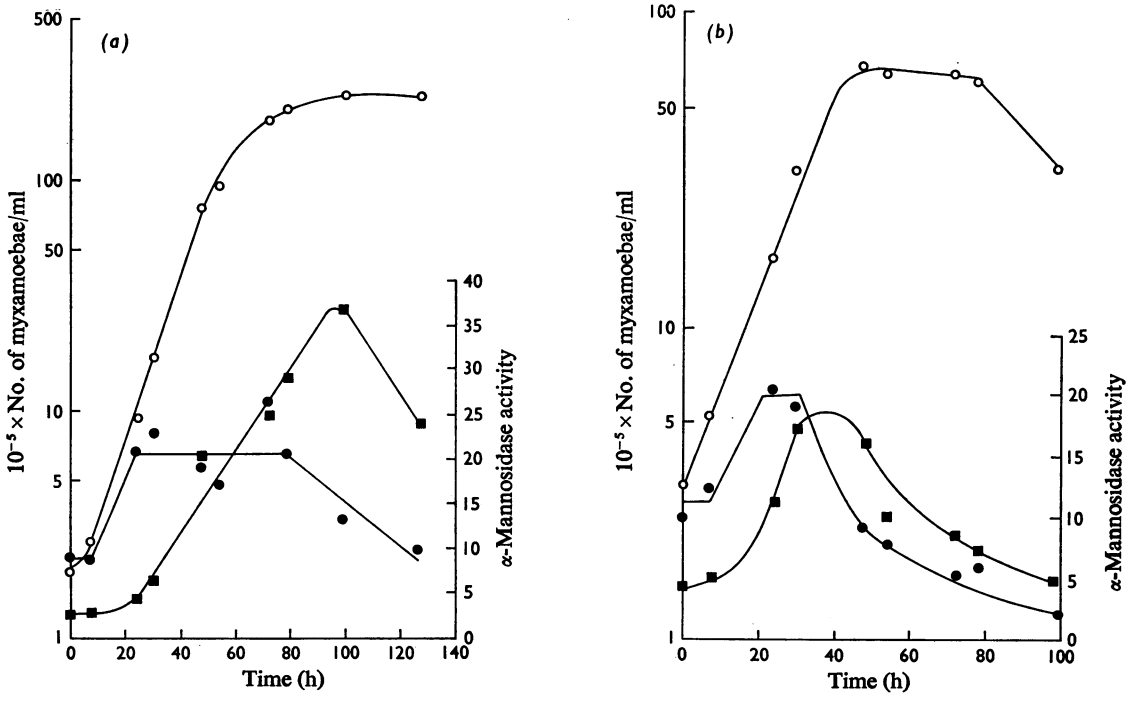

Fig. 2. *Synthesis of α-mannosidase during growth of myxamoebae of D. discoideum*

The myxamoebae were grown (*a*) on axenic medium+glucose and (*b*) on axenic medium without added carbohydrate. ○, Cell number; ●, specific activity of α-mannosidase in the myxamoebae (nmol/min per mg of protein); ■, α-mannosidase content of the medium (nmol/min per ml of medium).

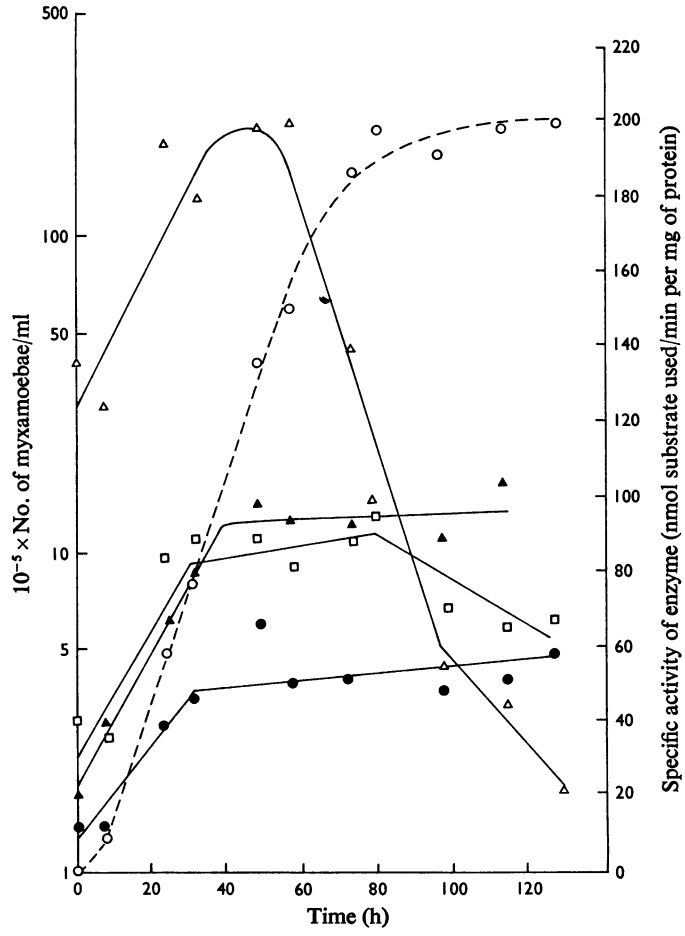

Fig. 3. *Synthesis of enzymes during growth of myxamoebae of D. discoideum on axenic medium+glucose*

○, Cell number; △, alkaline phosphatase (scale ×10); □, acid phosphatase; ●, β-glucosidase-1 (scale ×10); ▲, UDP-glucose pyrophosphorylase (scale ×2).

extent acid phosphatase, can also be found extracellularly in amounts that vary with the growth medium. In those cases (Figs. 1*b* and 2*b*) where, after prolonged shaking in the stationary phase, some of the cells lysed, all enzyme activities were detected extracellularly.

Intracellular localization of enzymic activities

It was reported that *N*-acetylglucosaminidase occurs in association with other acid hydrolases in lysosomal particles (Wiener & Ashworth, 1970). α-Mannosidase, alkaline phosphatase and, to a lesser extent, β-glucosidase were also found in this fraction of the cell (Table 2). It is not clear if the changes in intracellular distribution of these enzymes at different growth phases (Table 2) are significant. UDP-glucose pyrophosphorylase is predominantly a soluble enzyme, although a significant portion can be recovered in the microsomal fraction together with the remainder of the alkaline phosphatase (Table 3).

The alkaline phosphatase activity that occurred in the lysosomal fraction was not solubilized when the organelles were lysed by being suspended in water, unlike the acid hydrolase activities (Wiener & Ashworth, 1970). Instead the activity remained associated with the particulate membrane fragments. The activity was, however, obtained in a soluble form by treatment of these fragments and also the microsomal fraction with a non-ionic detergent such as Triton X-100 (Table 4). The intracellular distribution of these enzymes did not change significantly during

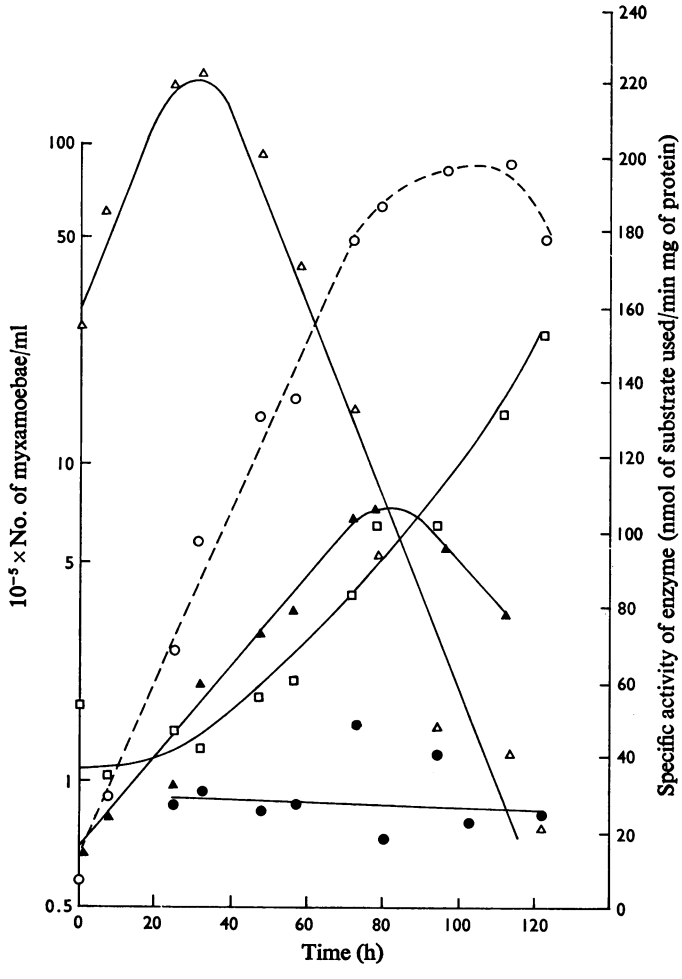

Fig. 4. *Synthesis of enzymes during growth of myxamoebae of D. discoideum on axenic medium without added carbohydrate*

○, Cell number; △, alkaline phosphatase (scale ×10); □, acid phosphatase; ●, β-glucosidase-1 (scale ×10); ▲, UDP-glucose pyrophosphorylase (scale ×2).

the growth cycle. The activities in the lysosomal and microsomal membrane preparations appeared to be different since they behaved differently when solubilized by detergents (Table 4). Detergent treatment solubilized more than 80% of the activity of the microsomal membranes, and at the same time doubled the total activity.

Discussion

Myxamoebae of *D. discoideum* strain NC-4 (which can only be grown on bacteria) were reported to contain little or no *N*-acetylglucosaminidase (Loomis, 1969*a*) or α-mannosidase (Loomis, 1970). Similarly, myxamoebae of *D. discoideum* strain Ax-2 grown on bacteria contained little of these two activities (Table 1). With both strains the specific activity of these two enzymes increases during the cell differentiation processes leading to fruiting body construction. Similar observations have been made with the other enzymes discussed in the present paper. Sussman & Sussman (1969), in a review of experiments of this type, have presented evidence that such increases represent protein synthesis *de novo* and are a consequence of selective gene expression during the development of *D. discoideum*. There is also independent genetic evidence that selective gene expression occurs during the development of *D. discoideum*

Table 2. *Lysosomal distribution of hydrolases*

The fractionation procedure is described in the Materials and Methods section.

Enzyme	% of enzyme present in lysosomal fraction after growth in			
	Axenic medium+glucose		Axenic medium	
Cell density at harvest ...	1×10^6–5×10^6	2×10^7	1×10^6–4×10^6	8×10^6
Acid phosphatase	66	81	72	84
N-Acetylglucosaminidase	69	86	67	89
α-Mannosidase	65	79	61	74
β-Glucosidase-1	41	59	44	46
Alkaline phosphatase	67	46	51	36

Table 3. *Intracellular distribution of enzymes*

Myxamoebae were grown in axenic medium+86mM-glucose and were harvested at 3.6×10^6 myxamoebae/ml. The myxamoebae were washed, resuspended in 0.25M-sucrose and fractionated as described. Specific activities refer to nmol of substrate/min per mg of protein.

Enzyme	Fraction					
	Lysosomal		Microsomal		Soluble	
	Specific activity	% of total	Specific activity	% of total	Specific activity	% of total
Acid phosphatase	161	72	138	18	12	10
N-Acetylglucosaminidase	850	78	68	12	33	10
Alkaline phosphatase	32	67	14	30	0.5	3
UDP-glucose pyrophosphorylase	9	4	50	19	86	77

(Loomis, 1969*b*). It has always been assumed that growth and cell differentiation are mutually exclusive phenomena, because differentiation of the myxamoebae is only observed after they have been removed from nutrients (Bonner, 1969) and, further, if nutrients (bacteria) are added to the intermediate stages of development then the cells can revert to the myxamoebal condition (Raper, 1940). Unexpectedly, myxamoebae of strain Ax-2 contained both α-mannosidase and *N*-acetylglucosaminidase (Table 1, Wiener & Ashworth, 1970) in amounts similar to the peak quantities reported to be present only in differentiating cells. It can be concluded from this observation either that growth and cell differentiation are not mutually exclusive phenomena or that the syntheses of these two enzymes are not part of the 'developmental programme' as defined by Sussman & Sussman (1969). Neither of these conclusions is particularly satisfactory. The evidence that cell differentiation and growth are mutually exclusive phenomena is very strong (Bonner, 1969; Loomis, 1969*b*) and these two enzymes also obey all the criteria (Loomis, 1969*a*,*c*) adopted for other developmentally regulated enzymes. If the synthesis of these enzymes is regarded as not being part of the 'developmental programme' then either the concept of such a 'programme' must be questioned or the term must be redefined to include events other than protein synthesis. If cell differentiation is a phenomenon distinct from growth it must involve the sequential and selective expression of genetic information in both space and time to achieve the highly specific morphogenesis, i.e. there must be a 'developmental programme'. One solution of this problem is to assume that the developmentally significant event is not the synthesis but the excretion of these two enzymes. In the case of *N*-acetylglucosaminidase this occurs as the myxamoebae cease true exponential growth, and in the case of the α-mannosidase, slightly before this time. Both these enzymes occur in the lysosomal fraction but, since other lysosomal enzymes are either not excreted, or if they are excreted, are not excreted to the same extent, this process must be specific. It is thus possible that the lysosomal fraction of the myxamoebae contains true lysosomal vesicles and also vesicles that contain enzymes in transit from their site of synthesis to the extracellular medium. It has been suggested (Wiener

Table 4. *Distribution of alkaline phosphatase*

Myxamoebae were grown on axenic medium+86mM-glucose and harvested at 3.6×10^6 myxamoebae/ml. The myxamoebae were washed and resuspended in 0.25M-sucrose and fractionated as described in the text. The lysosomal fraction was resuspended in water and incubated at 0°C for 30min. The membrane fragments (21 enzyme milliunits/ml) were collected by centrifugation at 100000*g* for 1h and resuspended in 0.25M-sucrose. Portions were treated with Triton X-100 [final concentration 0.2% (v/v)] and sodium dodecyl sulphate [final concentration 0.1% (w/v)] and recentrifuged to obtain a particulate and soluble fraction. The particulate fractions were resuspended in a volume of 0.25M-sucrose equal to the volume of the supernatant (soluble) fraction and both were assayed for alkaline phosphatase activity.

	Enzyme activity (milliunits/ml) in fractions from					
	'Lysosomal' membranes			'Microsomal' membranes		
Treatment	Soluble	Particulate	% yield	Soluble	Particulate	% yield
None	4	17	100	2.5	14.5	100
Triton X-100	15.5	7.2	110	29	5.7	205
Sodium dodecyl sulphate	6.3	16	105	33	2.0	205

& Ashworth, 1970) that the lysosomal fraction is heterogeneous and such heterogeneity provides, in a simple fashion, a mechanism whereby α-mannosidase and *N*-acetylglucosaminidase may be excreted selectively at a specific time (Figs. 1 and 2). There is also evidence (D. Every & J. M. Ashworth, unpublished work) that during the cell differentiation of myxamoebae grown on bacteria these enzymes are excreted. Thus the assumption that the excretion of these enzymes is part of the 'developmental programme' seems reasonable.

The synthesis of *N*-acetylglucosaminidase during the growth phase shows some unusual features. There is a doubling of the specific activity at the time (Fig. 1) that excretion begins, suggesting that myxamoebae of *D. discoideum* Ax-2 contain either one 'quantum' (equivalent to a specific activity of 200) or two 'quanta' (equivalent to a specific activity of 400) of this enzyme. It seems, therefore, that the control of the synthesis of *N*-acetylglucosaminidase is such that only a few characteristic specific activities are observed. A similar phenomenon has been observed by Newell & Sussman (1970) who studied the synthesis of UDP-glucose pyrophosphorylase during cell differentiation of *D. discoideum* NC-4. M. Sussman (personal communication) has suggested the term 'quantal control' for this phenomenon and Ashworth (1971) has shown, with a different strain of *D. discoideum*, that specific activities of *N*-acetylglucosaminidase of 560–630 (3 'quanta') and 900 (5 'quanta') may also be obtained under the appropriate conditions. Newell & Sussman (1970) showed that 'quantal control' of UDP-glucose pyrophosphorylase operates at the transcriptional level, but the significance of these observations is unknown.

Because it is possible to alter the relative amounts of various enzymes believed to be part of the 'developmental programme' by growing the myxamoebae axenically (Figs. 1–4) the nature of the 'developmental programme' can be tested. If this controls the time and amount of protein synthesis, and this only, and if the amount of enzyme activity present is the critical factor in successful morphogenesis then it must be expected that myxamoebae grown under different conditions would show altered morphogenetic behaviour and/or regulate their enzymic composition during morphogenesis so that they achieved that assembly of enzymes which was appropriate irrespective of their initial enzyme composition.

Thus it is possible to approach, experimentally, the problem raised in the preceding paper (Edmundson & Ashworth, 1972) of whether the acquisition of the appropriate enzyme activities, although a necessary condition of successful morphogenesis, is also a sufficient condition. The following paper discusses this question (Quance & Ashworth, 1972).

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References

Ashworth, J. M. (1971) *Symp. Soc. Exp. Biol.* in the press
Ashworth, J. M. & Sussman, M. (1967) *J. Biol. Chem.* **242**, 1966
Ashworth, J. M. & Watts, D. (1970) *Biochem. J.* **119**, 175
Bonner, J. T. (1969) *The Cellular Slime Molds*, 2nd. edn., p. 43, Princeton University Press
Coston, B. & Loomis, W. F. (1969) *J. Bacteriol.* **100**, 1208
Edmundson, T. D. & Ashworth, J. M. (1972) *Biochem. J.* **126**, 593

Loomis, W. F. (1969*a*) *J. Bacteriol.* **97**, 1149
Loomis, W. F. (1969*b*) *J. Bacteriol.* **99**, 65
Loomis, W. F. (1969*c*) *J. Bacteriol.* **100**, 417
Loomis, W. F. (1970) *J. Bacteriol.* **103**, 375
Lowry, G. A. & Conchie, J. (1966) *Methods Enzymol.* **8**, 571
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
Newell, P. C. & Sussman, M. (1970) *J. Mol. Biol.* **49**, 627
Quance, J. & Ashworth, J. M. (1972) *Biochem. J.* **126**, 609
Raper, K. (1940) *J. Elisha Mitchell Sci. Soc.* **56**, 241
Sussman, M. (1966) *Methods Cell Physiol* **2**, 397
Sussman, M. & Sussman, R. (1969) *Symp. Soc. Gen. Microbiol.* **19**, 403
Watts, D. & Ashworth, J. M. (1970) *Biochem. J.* **119**, 171
Wiener, E. & Ashworth, J. M. (1970) *Biochem. J.* **118**, 505