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The Metabolism of Macromolecules during the Differentiation of Myxamoebae of the Cellular Slime Mould *Dictyostelium discoideum* Containing Different Amounts of Glycogen

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1. Methods of obtaining myxamoebae of *Dictyostelium discoideum* strain Ax-2 (ATCC 24397) with glycogen contents in the range 0.05–5mg of glycogen/10^8 cells are described. The changes in cellular glycogen, protein and RNA content during the differentiation of such myxamoebae were determined. 2. Myxamoebal glycogen is not conserved during differentiation and gluconeogenesis may occur even in cells that contain a large amount of glycogen initially. 3. There is a marked net loss of cellular protein and RNA during differentiation and associated with this there are also marked decreases in the sizes of the intracellular pools of amino acids, acid-soluble proteins and pentose-containing materials. 4. During the early stages of development some protein and pentose(s) are excreted, but this cannot account for the decreased cellular content of protein and RNA. 5. There is a linear rate of production of NH_3 during development, and oxidation appears to be the fate of the major portion of the degraded protein and RNA. 6. However, provision of an alternative metabolizable energy source (glycogen) has little effect on the rate or extent of protein or RNA breakdown or on the changes in the sizes of the intracellular pools of amino acids, acid-soluble proteins and pentose-containing materials. 7. It is concluded that during development there is a requirement for the destruction of specific RNA and protein molecules for reasons other than the provision of oxidizable substrates. 8. The kinetic model of Wright *et al.* (1968) is discussed in relation to these changes in macromolecular content.

One of the advantages that microbial organisms possess for biochemical investigations is that they can frequently be grown in media of different chemical composition. The cellular slime mould *Dictyostelium discoideum* exists in the feeding, vegetative state as independent amoeboid cells (myxamoebae), and it has been shown that myxamoebae of a mutant strain, Ax-2, can be grown in media which differ in carbohydrate content (Watts & Ashworth, 1970) and that changes in chemical composition (Weeks & Ashworth, 1972), enzyme composition (Ashworth & Quance, 1972) and physiological behaviour (Ashworth & Watts, 1970) can be thereby induced. When such myxamoebae are removed from nutrients and placed on a moist, solid surface they aggregate together to form a multicellular and macroscopic entity, the slug or grex, the component cells of which become either the stalk or the spore cells of the fruiting body (sorocarp), whose formation marks the end of the cell-differentiation phase of the life cycle (Bonner, 1967). Since growth and cell differentiation are mutually exclusive phenomena in this organism, changes can be induced in the cells during the growth phase, and the purely developmental consequences of these changes can be studied subsequently. This is equivalent to studying the differentiation of cell populations containing identical genomes expressing their developmental potential in metabolically different cellular environments, and allows us to distinguish between developmentally important events and changes that may occur during development but which are not essential for successful development (Quance & Ashworth, 1972).

However, little advantage has been taken of this aspect of the life cycle of *D. discoideum* and most workers have concentrated on describing the events which occur during the differentiation of myxamoebae grown on bacteria as source of nutrients. As a consequence of such studies Sussman & Sussman (1969) concluded that there is little net change in carbohydrate content of the cells during development, but that considerable qualitative changes occur in the nature of the carbohydrates which the cells contain at different stages of development (White & Sussman, 1961). Thus gluconeogenesis has been

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assumed to be an activity of minor significance (Cleland & Coe, 1969; Sussman & Sussman, 1969) and the energy necessary to interconvert the various carbohydrates and to provide for the many other metabolic requirements of the cell, has been assumed to come from the massive net loss in cellular protein, RNA and dry weight (Gregg & Bronsweig, 1956; Wright & Anderson, 1960; White & Sussman, 1961). Mathematical models which simulate these changes have been constructed (Gustafson & Wright, 1972; Wright, 1973) and used to predict 'critical variables' for reactions essential to differentiation. An assumption underlying this approach is that the metabolic changes modelled are necessarily part of the morphogenetic programme and that these 'critical variables' for the metabolic changes correspond to 'critical variables' for differentiation. It is the purpose of the present paper to demonstrate that there can be massive net changes in the carbohydrate content of the cells of *D. discoideum* during development, that gluconeogenesis can play a vital role in such development and that the massive losses in cellular protein and RNA occur for reasons other than the provision of energy.

Experimental

Materials

Trehalose, bovine serum albumin, ribose, glycine, ninhydrin and deoxyribose were obtained from Sigma (London) Chemical Co. Ltd., London W.5, U.K.; phosphoglucomutase, glucose 6-phosphate dehydrogenase, glutamate dehydrogenase and 6-phosphogluconate dehydrogenase were from Boehringer Corp. (London) Ltd., London W.5, U.K.; *NN*-dioctylmethylamine was from Koch–Light Laboratories, Colnbrook, Bucks., U.K.; [U-^{14}C]-glucose was from The Radiochemical Centre, Amersham, Bucks., U.K.; Amberlite Monobed MB3 Resin was from BDH Chemicals Ltd., Poole, Dorset, U.K. Orcinol was obtained from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

All other chemicals were of the highest purity commercially available and purchased either from the sources described by Hames *et al.* (1972) or from BDH Chemicals Ltd.

Methods

Growth and differentiation of myxamoebae. Myxamoebae of strain Ax-2 (A.T.C.C. 24397) were grown, harvested, and washed as described by Weeks & Ashworth (1972). The washed myxamoebae were deposited on well-washed Millipore filters at cell densities of 2.5×10^7–5.0×10^7 myxamoebae/filter, supported by cellulose filter-paper pads saturated with 1.6ml of buffer containing 1.5g of KCl/litre, 0.5g of $MgCl_2\cdot6H_2O$/litre and 0.5g of streptomycin sulphate in 50mM-phosphate buffer, pH6.5/litre. Development was then allowed to occur at 22°C in a dark humid atmosphere and fruiting bodies were formed 24±2h after deposition of the myxamoebae (Sussman, 1966).

Calculation of cellular concentrations of materials. To correct for variable cell recoveries from Millipore filter supports during development, the values for cellular protein, RNA and glycogen contents were corrected to amounts/10^8 cells by measuring, in the same sample, the amount of DNA and assuming that (1) the DNA content of 10^8 myxamoebae is 17.86μg/10^8 cells and (2) that the DNA content/cell does not change during development. There is evidence that both these assumptions are valid (Hames, 1972).

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 4ml of water. These samples were stored at −35°C. After being thawed, samples were made up to 5ml with water and sonicated with a 100W MSE ultrasonic disintegrator (peak-to-peak amplitude 9μm) for either four 15s periods (samples 0–16h) or for twelve 15s periods (samples 16–30h) 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 *et al.* (1951) with bovine serum albumin as the standard. Cold 50% (w/v) trichloroacetic acid (1ml) was added to another 4ml of extract and the mixture was incubated for 1h at 0°C. The resulting precipitate was centrifuged (1000*g*, 20min). The pellet was washed with 2ml of cold 10% (w/v) trichloroacetic acid and the supernatants from the two centrifugations pooled and used for assay of cold acid-soluble protein by the method of Lowry *et al.* (1951). The pellet was resuspended in 2.5ml of 5% (w/v) trichloroacetic acid and heated at 90°C for 30min to hydrolyse the DNA and RNA. The hydrolysate was centrifuged (1000*g*, 20min) and the supernatant (hot-acid-soluble fraction) assayed for DNA by the modified method of Giles & Myers (1965) as described by Hames *et al.* (1972), RNA by the orcinol reaction (Mejbaum, 1939) and protein by the method of Lowry *et al.* (1951). The acid-soluble pellet was dissolved in 4ml of 1M-NaOH and assayed for protein by the method of Lowry *et al.* (1951).

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

Determination of cellular glycogen content. The glycogen content of cells was determined as described by Hames *et al.* (1972) and expressed as mg of glucose equivalents/10^8 cells.

Determination of total cellular carbohydrate content. At various times during differentiation, cells were harvested into ice-cold water, and stored at −35°C. After being thawed, the cell suspensions were made up to 10ml with water, and portions (5ml) were sonicated with a 150W MSE ultrasonic disintegrator (peak-to-peak amplitude of 10μm) for either four 15s periods (samples 0–16h) or for twelve 15s periods (samples 16–30h), with continuous cooling in an ice–salt bath to prevent excessive heating. Samples prepared in this fashion were then assayed for hexose by the anthrone method of Hassid & Abraham (1957).

Determination of cellular amino acid. At various times during the developmental phase, cells were harvested into ice-cold water to give approx. 2.0×10^8 cells/4ml of water. These samples were stored at −35°C. Cell extracts were prepared by sonication as above and amino acids were extracted from 4ml of the sample by boiling for 30min. After incubation at 0°C for 15min, insoluble cell debris was removed by centrifugation (1000*g*, 10min) and the supernatant was analysed for amino acid by the ninhydrin method (Mitchell & Moyle, 1953). NH_3 in the samples was responsible for some of the ninhydrin reaction, and was corrected for by assay of NH_3 by the method of Levitzki (1971) and the subtraction of the ninhydrin colour given by that amount of NH_3 present in the sample, from the total sample ninhydrin colour. The standard solutions were prepared from glycine and NH_4Cl.

Determination of trichloroacetic acid-soluble pentose material. Trichloroacetic acid-soluble fractions were prepared as described above and assayed for pentose by using the orcinol reaction (Mejbaum, 1939).

Determination of extracellular protein, u.v.-absorbing material and ribose. Myxamoebae were allowed to develop as described above except that the buffered salts solutions used to keep the Millipore filters moist did not contain streptomycin since this reacts in the assay for protein (Lowry *et al.*, 1951). At various times during the developmental phase, the buffered salt solution was harvested from eight Millipore filter supports by squeezing the supporting pads with protective gloves. The collected buffer was centrifuged (1000*g*, 10min) to remove cellulose fibres and then stored at −35°C. After thawing, the protein content was determined by the method of Lowry *et al.* (1951) with anhydrous bovine serum albumin as standard. Other samples of the buffer were used to determine the E_{260} and E_{280}, and to assay the ribose content by the orcinol reaction (Mejbaum, 1939) with ribose as standard. For all these assays, correction was made for the reaction or absorbance given by the components of the buffer itself.

Determination of NH_3. Myxamoebae were allowed to differentiate on Millipore filter supports (27mm diam.) in closed Conway dishes. At intervals during the developmental phase, the buffered salts solution was collected by squeezing the Millipore membrane-supporting pads, centrifuged (1000*g*, 10min) to remove cellulose fibres, and stored at −35°C. After slow thawing, the buffer was assayed for NH_3 by using glutamic dehydrogenase as described by Levitzki (1971) and correcting for the initial NH_3 content.

Growth and development of [U-^{14}C]glucose-labelled myxamoebae. Myxamoebae of *D. discoideum* strain Ax-2 were grown in 80ml of axenic medium containing [U-^{14}C]glucose (25μCi) and a final glucose concentration of 86mM.

Cultures were shaken in 250ml Erlenmeyer flasks on a rotary shaker at 150–170rev./min until the cell density approached 10^7 cells/ml (for cells in medium containing added glucose) and 6×10^6 cells/ml (for cells in medium lacking added glucose). The myxamoebae were harvested and allowed to develop at a density of 7.5×10^6 myxamoebae/27mm diam. 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 1ml of 1M-NaOH to trap any $^{14}CO_2$ released. Control dishes contained Millipore filter supports without cells.

Analysis of [U-^{14}C]glucose-labelled myxamoebae. Cell-free extracts of myxamoebae were obtained as described above. Samples of vol. 1ml of these extracts were added to 10ml 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 4ml of water, and samples of this added to 10ml of Triton–toluene scintillation fluid. Protein was isolated from cell-free extracts by addition of 1ml of 30% (w/v) trichloroacetic acid to 2ml of extract, and then collection of the precipitated protein by centrifugation after incubation at 0°C for 30min. The crude protein precipitate was dissolved in 1ml of 1M-NaOH and insoluble material was removed by centrifugation. This material was re-extracted with 1ml of 1M-NaOH and the alkaline extracts were pooled and incubated at 0°C for 30min with 1ml of 80% (w/v) trichloroacetic acid to reprecipitate protein. This process was repeated twice more and the final, purified, protein precipitate was dissolved in 1ml of 1M-NaOH. Samples of this were taken for determination of the radioactive content by the method 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 for radioactivity 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 buffer solution used to keep the Millipore filter moist was also collected, by squeezing the Millipore filter-support pads with protective gloves, centrifuged to remove cellulose fibres (1000*g*, 10min) and then samples were counted for radioactivity by the method of Fox (1968).

The 1M-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).

Development of myxamoebae grown in the presence of [U-^{14}C]aspartate. Myxamoebae were grown in portions (80ml) of axenic medium containing [U-^{14}C]aspartate (3μCi) either in the absence or presence of glucose (86mM final concentration). The myxamoebae were harvested, allowed to develop and analysed at various stages of development as described above for [U-^{14}C]glucose-grown myxamoebae. The buffered salts solution 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 10ml of a Triton X-100–toluene mixture (Fox, 1968) and counted for radioactivity in a Packard Tricarb scintillation spectrometer as described above.

Determination of the specific radioactivity of trehalose and glucose. Cells were harvested at various times during the developmental phase and extracts prepared as described above. A sample of extract (4.5ml) was added to 0.25ml of 60% (w/v) perchloric acid and the mixture was incubated for 30min at 0°C. The precipitated protein was removed by centrifugation (1000*g*, 20min), the supernatant neutralized with KOH, and the resulting precipitate of potassium perchlorate also removed by centrifugation. Saturated Na_2SO_4 (0.25ml) and ethanol (10ml) were added to 5ml of neutralized perchloric acid extract and the mixture incubated at 0°C for 15min. The precipitated glycogen was removed by centrifugation (38000*g*, 15min) and the supernatant deionized by passage through Amberlite Monobed MB3 resin. The resin was washed with an equal volume of water, and the pooled solutions were then carefully evaporated to dryness under vacuum at 50°C, and the residue resuspended in 5ml of water. This solution was further deionized by passage through Monobed MB3 resin which was washed as before. The pooled solutions were evaporated to dryness at 50°C under vacuum by using a Rotary Evapomix (Buchler Instruments Ltd., New York, U.S.A.), then resuspended in 0.1ml of water and spotted on to Whatman no. 4 chromatography paper. The chromatogram was developed with propan-2-ol–butan-1-ol–water (140:20:40, by vol.) and carbohydrates were detected by using alkaline $AgNO_3$ (Trevelyan *et al.*, 1950) and sodium metaperiodate (Evans & Dethier, 1957), and then identified by comparison with authentic samples run on the same paper. Trehalose and glucose were eluted as described by Dimler *et al.* (1952), concentrated at 50°C with a Rotary Evapomix and then each redissolved in 2ml of water. Samples were assayed for trehalose and glucose (as described below) and for radioactivity by the method of Fox (1968).

Determination of trehalose. Trehalase was prepared from *Neurospora crassa* by a modification of the method of Hill & Sussman (1963) as described by Hames (1972). The enzyme was specific for trehalose when tested on a wide range of carbohydrates including *D. discoideum* glycogen, galactose, maltose, cellobiose, and cellulose (Hames, 1972).

Cells were harvested at various stages of development into ice-cold water, extracts prepared as described above, and then 0.25ml of ice-cold 60% (w/v) perchloric acid immediately added to 4.5ml of the cell extract. The mixture was incubated at 0°C for 30min, and the protein precipitate was then removed by centrifugation (1000*g*, 20min). The supernatant was neutralized with KOH, then the precipitate of potassium perchlorate was removed by centrifugation (1000*g*, 10min) and the supernatant assayed for trehalose. Each assay mixture contained 0.8ml of neutralized perchloric acid extract, 0.1ml of 0.5M-sodium potassium phosphate buffer, pH5.6, and 0.1ml of purified trehalase. This was incubated for 4h at 37°C, boiled for 10min to stop the reaction, and then the glucose content of the solution was determined by the hexokinase method of England & Randle (1967). This value was corrected for endogenous glucose by subtraction of the glucose content of an extract treated as above but omitting the incubation with trehalase.

Determination of specific radioactivity of cell-wall polysaccharide. Mature fruiting bodies (40h developmental time) were harvested into water and sonicated as described above. Cell extract (2ml) was added to 1ml of 90% (w/v) KOH and the suspension heated at 100°C for 20min. Insoluble material was removed by centrifugation at 17000*g* for 10min and extracted with 1ml of 30% (w/v) KOH at 100°C for a further 20min. After re-centrifugation, the pellet was washed twice with 2ml of water each time. Some samples were filtered through Sartorius membrane filters (25mm diam.; cat. no. 11306), washed twice with 5ml of water, dried under an infrared lamp, and counted for radioactivity in 10ml of toluene scintillation mixture. Other samples were dissolved in 67% (w/v) H_2SO_4 (2h at room temperature) 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 by using the channels-ratio method of Baillie (1963).

Assay of phosphorylase. Phosphorylase activity was assayed by the method of Firtel & Bonner (1972) with cell extracts prepared in water and stored frozen at −35°C, except that assay mixtures contained 0.1 unit of 6-phosphogluconate dehydrogenase and the assay was performed at 23°C.

Assay of amylase. Amylase activity was assayed by the method of Jones & Wright (1970), but with some modifications.

Reaction mixtures contained the following in a total volume of 0.3ml: buffer [either 3.0μmol of sodium acetate, pH4.8, or 30μmol of imidazole–HCl, pH6.9], 1.0μmol of NaCl, 2.0mg of glycogen and 0–0.2ml of cell extract (prepared as described above). Control reactions containing cell extract but no glycogen were used to correct for reducing sugar present in the extract, and reaction mixtures containing maltose (0–1.2μmol) but no enzyme were used to prepare a calibration curve.

The reaction was done at 23°C for 60min, and then 0.2ml of colour reagent [1% 3,5-dinitrosalicylic acid–30% (w/v) potassium sodium tartrate in 0.4M-NaOH] was added. The reaction tubes were then heated at 100°C for 5min, cooled and 2.0ml of water added to each. After centrifugation (1000*g*, 10min) to remove any insoluble material, the E_{540} of the supernatant was measured. The rate of production of reducing sugar was linear with respect to the amount of extract used or with the time of incubation up to at least 60min.

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/filter, as described above. At intervals during the developmental phase, the cells from five filters were harvested into 3ml of ice-cold water and stored at −15°C. After thawing the cell suspensions slowly in the cold, cell extracts were prepared as described above, and assayed for maltase activity as described below.

Assay mixtures contained 80μmol of sodium phosphate–sodium citrate buffer, pH4.0, 2mg of maltose, 0.1ml of cell extract, made up to a final volume of 1ml with water, and incubated at 23°C for 1h. The reaction was terminated by boiling the assay mixture for 10min and glucose was 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 the cell extracts and maltose solution respectively.

Electron microscopy of myxamoebae. Myxamoebae, harvested and washed and resuspended in water as described above, were centrifuged at 2000*g* for 10min at 0°C and the cellular pellet was resuspended in a solution of 2% (w/v) glutaraldehyde (in 0.2M-sucrose–10mM-Tris–HCl buffer, pH7.6) for 2h at 0°C. The fixed material was collected by centrifugation, post-fixed in buffered 2% (w/v) osmic acid (30min), dehydrated through an ethanol series and infiltrated with propylene oxide and Araldite, which was then allowed to polymerize for 2 days at 60°C. Silver and grey sections were cut on an L.K.B. Ultratone III microtome and stained with uranyl acetate.

The sections were examined in a Siemens Elmiskop 1A electron microscope at an accelerating voltage of 80kV with a 200μm condenser aperture.

Results

D. discoideum myxamoebae, strain Ax-2 (ATCC 24397), grow in the basic axenic medium with a mean generation time of approx. 10h, but this can be decreased to 8h by the addition of glucose to the medium to a final concentration of 0.086M (Watts & Ashworth, 1970). If the glucose concentration is increased beyond 0.1M, decreased growth rates and cell yields result (Table 1).

Myxamoebae grown in the absence of added glucose contain low but variable concentrations of glycogen; over a three-year period values in the range of 0.046–0.310mg of glycogen/10^8 cells were obtained. The source of this variation is the Oxoid yeast extract

Table 1. *Growth of Dictyostelium discoideum in axenic medium containing various concentrations of glucose*

Results are given as means ±S.E.M. with the number of determinations in parentheses.

Glucose concentration in medium (mM)	Cell doubling time (h)	Cell yield (×10^6 cells/ml)	mg of glucose equivalents/10^8 cells of	
			Glycogen	Carbohydrate
0	10.5*	8.30±0.74 (5)	0.11±0.02 (12)	0.27±0.03 (6)
86	8.0*	15.30±0.55 (9)	1.69±0.18 (8)	1.68±0.10 (5)
100	9.83±0.33 (3)	20.00±0.00 (2)	1.70±0.20 (2)	1.86±0.026 (5)
200	13.67±0.55 (9)	9.96±2.63 (5)	2.78±0.07 (3)	2.98±0.07 (3)
300	19.67±0.33 (3)	5.25±0.75 (2)	4.77±0.49 (4)	5.29±0.33 (4)
400	29.50±1.99 (5)	1.18±0.35 (4)	4.25±0.25 (2)	4.58±0.08 (2)

* Data taken from Ashworth & Watts (1970).

used in the medium. Addition of glucose to the axenic medium increases the myxamoebal glycogen content in exponentially growing myxamoebae in a linear manner over the range 0–0.3M final glucose concentration in the medium (Table 1), but further addition of glucose fails to increase the myxamoebal glycogen content beyond approx. 5.0mg of glycogen/10^8 cells and so this appears to be the maximum myxamoebal glycogen content obtainable by this method.

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 0.086M-glucose there is an increase in the cellular glycogen content when the growth rate begins to decrease at the end of the exponential-growth phase (Weeks & Ashworth, 1972). 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 contain added glucose.

The studies in the present paper have used myxamoebae with high glycogen contents obtained by both of the above methods. Although no differences have been observed that are attributable to the source of these cells, the second method described is preferred since greater cell yields are possible within much shorter time-periods. Myxamoebae develop normally within 24±2h irrespective of their glycogen content.

Cellular location of myxamoebal glycogen

Electron microscopy of myxamoebae containing large amounts of glycogen reveals large diffuse areas of electron-transparent material in the cytoplasm which are absent in myxamoebae containing low concentrations of glycogen (Plate 1). These areas, which are unbounded by membranes, are believed to be sites of myxamoebal glycogen storage.

Glycogen content of cells during differentiation

Myxamoebae grown in the absence of added glucose contain very low concentrations of glycogen and this glycogen is rapidly degraded during the first 4h of development (Hames *et al.*, 1972). Glycogen is then synthesized during the late-aggregation and slug-migration stages (5–15h) and finally broken down at the time of end-product saccharide synthesis. The amount of glycogen synthesized during this 5–15h period is considerably greater than the vegetative myxamoebae glycogen content and synthesis occurs when the cellular pool of glucose remains at a constant low concentration (Fig. 1), suggesting that gluconeogenesis is occurring. This is confirmed by the finding that the total cellular carbohydrate content, after an initial decrease presumably due to myxamoebal glycogen degradation, doubles during the developmental phase (Fig. 2).

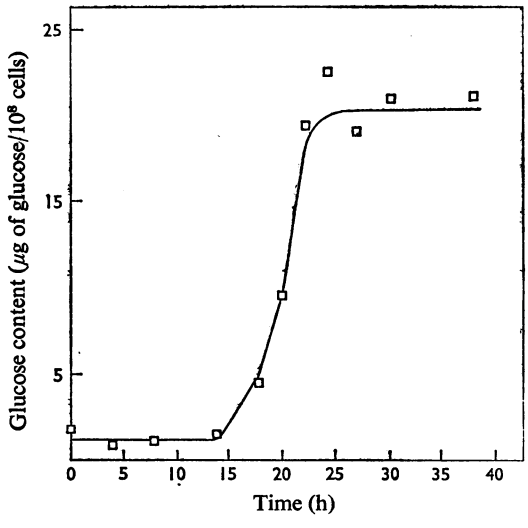


Fig. 1. *Changes in the glucose content of cells during the development of axenically grown myxamoebae containing* 0.13*mg of glycogen/*10^8 *cells initially*

For details see the text.

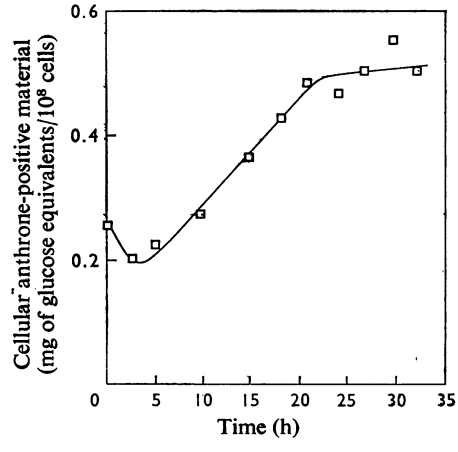
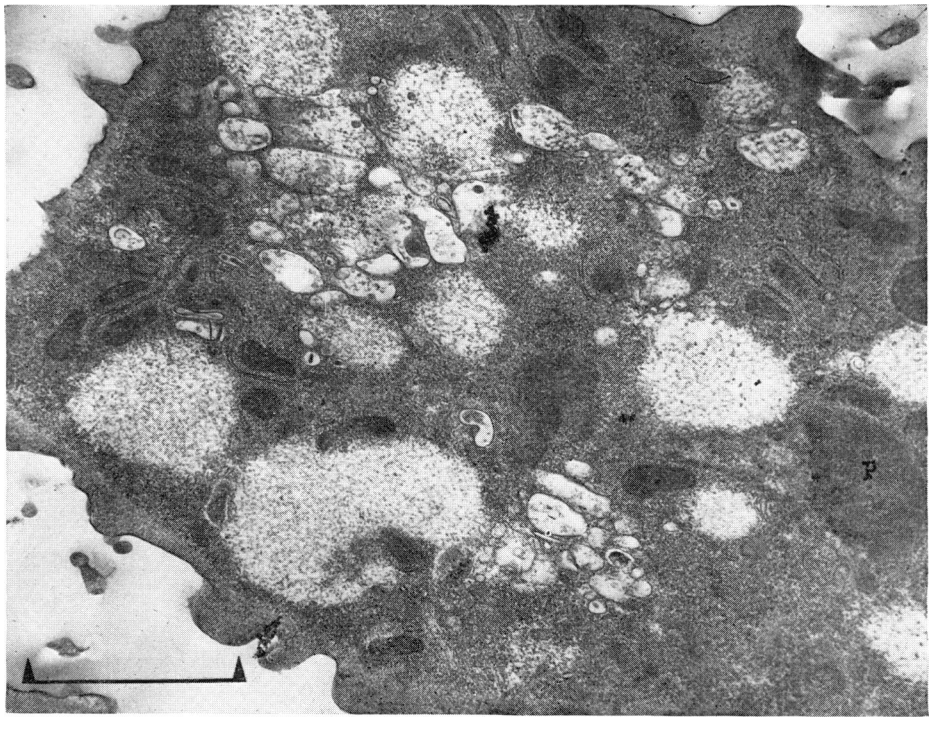


Fig. 2. *Changes in the carbohydrate content of cells (anthrone-positive material) during the development of axenically grown myxamoebae containing initially* 0.074*mg of glycogen/*10^8 *cells*

For details see the text.



EXPLANATION OF PLATE 1

Electron micrograph of a section of a myxamoeba of D. discoideum Ax-2 containing 4.64×10^{-8} *mg of glycogen*

The large, diffuse areas of electron-transparent material which are not bounded by a membrane are not seen in analogous sections of myxamoebae containing less than approx. 1×10^{-8} mg of glycogen. Bar represents 0.5 μm.

Myxamoebae initially containing more than 1mg of glycogen/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 (Hames *et al.*, 1972).

Fate of degraded glycogen

Myxamoebae initially containing more than 1mg of glycogen/10^8 cells exhibit a large loss of total cellular carbohydrate during development (Fig. 3). Since at least 90% of the anthrone-positive material of myxamoebae grown in the presence of glucose can be accounted for by their glycogen content (Ashworth & Watts, 1970; Table 1), this suggests that the major part of the myxamoebal glycogen is metabolized to non-hexose materials. 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 2). We realize that the growth of cells in [U-^{14}C]glucose-containing medium will label cellular components other than glycogen and protein which may be oxidized to CO_2 during development (e.g. ribose of RNA), but most of the cellular radioactivity lost during development of such cells is due to glycogen degradation and almost all of the radioactivity lost can be trapped as CO_2. The gas was shown to be CO_2 by mass spectroscopy (Hames, 1972). Measurements of the radioactivity

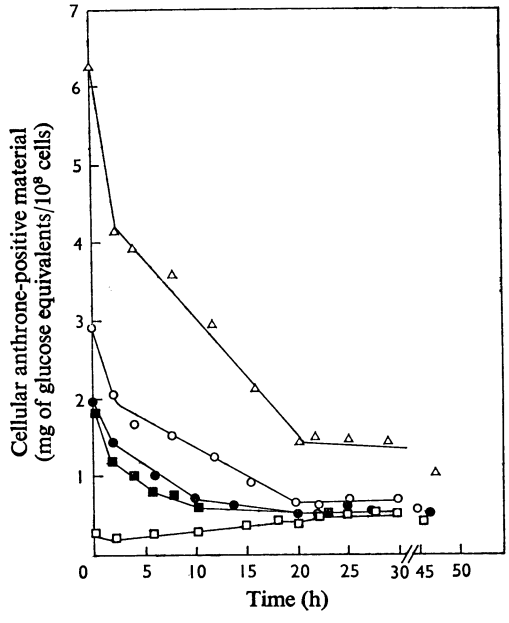


Fig. 3. *Changes in the carbohydrate content of cells (anthrone-positive material) during the development of axenically grown myxamoebae*

Myxamoebae were grown in axenic medium and contained initially, per 10^8 cells: 0.074mg of glycogen (□), 1.49mg of glycogen (■), 1.98mg of glycogen (●), 2.67 mg of glycogen (○) and 5.56mg of glycogen (△). For details see the text.

Table 2. *Oxidation of [U-^{14}C]glycogen, synthesized by myxamoebae during the growth phase, during their subsequent differentiation*

Myxamoebae were grown in the presence of [U-^{14}C]glucose and contained 6.26mg of glycogen/10^8 cells initially. All radioactivities are corrected for quenching as described in the text.

Sample (material and development time)	Total radioactivity (c.p.m./10^8 cells)	Cellular content of material (μmol/10^8 cells)	Specific radioactivity (c.p.m./μg-atom of carbon)
Total cells			
0h	551357	—	—
150h	139239	—	—
Cellular glycogen			
0h	398000	34.78	1907
33h	63017	5.75	1825
150h	23072	1.86	2071
Cellular protein			
0h	123500*	101.5*	243
33h	51200*	35.8*	286
150h	35500*	28.0*	253
CO_2			
150h	369056	—	—
Pad buffer			
150h	12083	—	—

* These values are estimates based on: (1) the radioactivity of cellular protein insoluble in trichloroacetic acid; (2) the total protein content of cell extracts (and thus do not take account of the radioactivity of acid-soluble protein); and (3) the assumption that the average molecular weight of the amino acids in *D. discoideum* protein is 100.

Table 3. *Relation between myxamoebal glycogen content and the sorocarp content of non-glycogen anthrone-positive material*

Myxamoebae containing different amounts of glycogen were allowed to differentiate for 30h and the amount of non-glycogen anthrone-positive material in the resulting sorocarps (fruiting bodies) was determined. Results are given as means ±S.E.M. with the number of experiments given in parentheses.

Myxamoebal glycogen content ($mg/10^8$ cells)	Sorocarp content of non-glycogen anthrone-positive material (mg of glucose equivalents/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)

of the pad buffer at the end of development indicate no significant accumulation of glycogen-degradation products.

However, apparently not all of the myxamoebal glycogen need be oxidized to CO_2 since comparison of the glycogen and total carbohydrate contents of mature sorocarps indicates that myxamoebae initially containing very high concentrations of glycogen produce fruiting bodies which contain more non-glycogen carbohydrate than those produced by myxamoebae initially containing low glycogen contents (Table 3).

Mechanism of glycogen degradation

The data described above indicate that myxamoebal glycogen is rapidly degraded during development, irrespective of myxamoebal glycogen content, to CO_2. During the early stages of development, axenically-grown myxamoebae possess high activity of amylase with a pH optimum of 4.8 (Wiener & Ashworth, 1970) (Fig. 4). Since cellular glycogen phosphorylase activity is extremely low at this time (the average specific activity of phosphorylase during the first 6h of development of cells containing either high or low amounts of glycogen is 0.075nmol of glucose 1-phosphate formed/min per mg of protein; Fig. 5), myxamoebal glycogen degradation must be due to amylytic and not phosphorolytic action. It is not clear whether the cellular amylase responsible is of the α or β specificity, but in the event of significant maltose production this sugar would be quickly hydrolysed to glucose by cellular maltase (Table 4). Thus glucose must be the main product of myxamoebal glycogen degradation and, as we have described previously, this is then oxidized to CO_2.

However, even though cellular amylase(s) and maltase are capable of degrading large amounts of

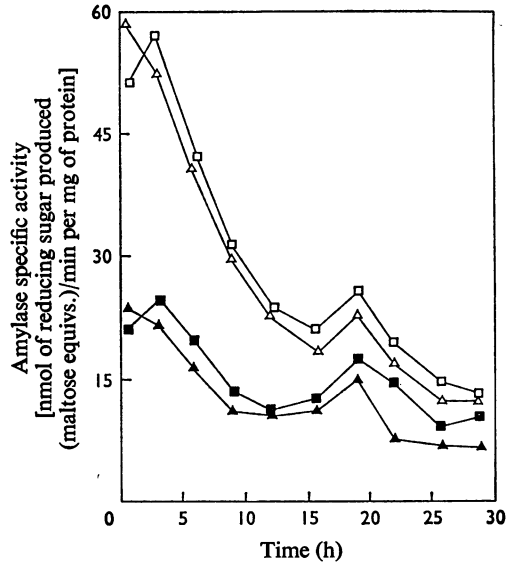


Fig. 4. *Variation in apparent amylase activity during the development of axenically grown myxamoebae*

Assays were carried out at pH4.8 (□, △) or pH6.9 (■, ▲)on cells derived from myxamoebae containing initially per 10^8 cells: either 0.27mg of glycogen (□, ■); or 7.5 mg of glycogen (△, ▲). For details see the text.

myxamoebal glycogen *in vivo* (Fig. 3), glycogen phosphorylase activity increases dramatically during fruiting-body construction. The apparent peak of amylase activity observed at 20h developmental time (Fig. 4) is probably due to this phosphorylase activity since although amylase activity is greatly decreased at pH7.0, the peak activity at 20h is not (Fig. 4). (Phosphorylase activity would be detected by the assay for amylase since axenically grown myxamoebae possess a phosphatase activity throughout development which reacts with the glucose 1-phosphate produced by phosphorylase action.) An explanation of these observations is that glycogen phosphorylase is needed to degrade a pool of glycogen which is, for some reason, not degraded by existing cellular amylase(s), but whose degradation is essential for successful development.

A noteworthy observation is that during the development of axenically grown myxamoebae, the peak specific activity of glycogen phosphorylase appears to be a function of the myxamoebal glycogen content such that myxamoebae initially containing 6.87±0.91mg of glycogen/10^8 cells accumulate approximately twice the amount of phosphorylase as myxamoebae initially containing 0.32±0.06mg of glycogen/10^8 cells (Fig. 5). That the

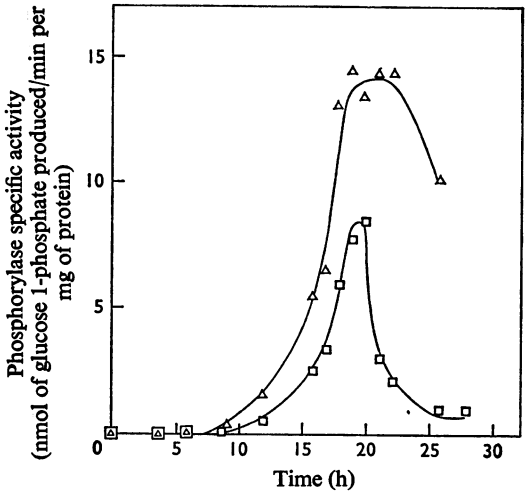


Fig. 5. *Changes in glycogen phosphorylase activity during development of axenically grown myxamoebae*

Myxamoebae were grown in axenic medium and contained initially, per 10^8 cells: either 0.30mg of glycogen (□); 5.35mg of glycogen (△). For details see the text.

Table 4. *Maltase activity of axenically-grown cells during the developmental stage*

Myxamoebae initially contained either 0.29mg of glycogen 10^8 cells (Expt. 1) or 5.85mg of glycogen/10^8 cells (Expt. 2) and were treated as described in the Experimental section.

Developmental time (h)	Cellular maltase activity (nmol of glucose produced/min per mg) Expt. 1	Expt. 2
0	20.50	19.38
11	9.55	6.34
22	7.20	4.48

difference in phosphorylase activity between these two sets of cells is not due to differences in the intracellular concentrations of enzyme activators or inhibitors is shown by the fact that the activities of mixtures of cell extracts are the sums of the activities displayed by the separate extracts. 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.

Use of glycogen to synthesize end-product saccharides

Myxamoebae were grown in [U-^{14}C]glucose-containing medium to label their myxamoebal glycogen uniformly and then allowed to develop. The fruiting bodies were harvested, the cellulose, trehalose and glucose which they contained were isolated and the specific radioactivities of these compounds were determined (Table 5). These experiments show that even though myxamoebal glycogen is degraded and radioactivity finds its way into end-product saccharides, the specific radioactivity of the latter is only 50–70% of that of the myxamoebal glycogen. Thus a source of carbon, other than myxamoebal glycogen, is used to synthesize at least part of the end-product saccharides even under conditions where there is at all times a large amount of glycogen present.

Table 5. *Use of [U-^{14}C]glycogen, synthesized by myxamoebae during the growth phase, as a precursor for end-product saccharide synthesis during the subsequent differentiation of such myxamoebae*

Myxamoebae were grown in the presence of [U-^{14}C]-glucose and initially contained 6.10mg of glycogen/10^8 cells (Expt. 1) or 5.25mg of glycogen/10^8 cells (Expt. 2). They were allowed to develop and glycogen and protein isolated and analysed as described in the Experimental section.

Compound	Specific radioactivity (c.p.m./μg-atom of carbon) Expt. 1	Expt. 2
Cellular glycogen		
0h	1820	1806
40h	—	1910
Cellular protein	188	257
40h	—	274
Cell-wall polysaccharide	1120	1110
Trehalose	—	927
Glucose	—	1251

Protein metabolism during development

During the development of axenically grown myxamoebae the amino acid pool (containing 20μmol of glycine equivalents of ninhydrin-positive material/10^8 cells initially) falls exponentially during development and reaches a value of 5–10μmol of glycine equivalents of ninhydrin-positive material/10^8 cells at the time of fruiting-body construction. Acid-insoluble protein is also degraded (Fig. 6), but regression analysis showed that the rate does not follow an exponential decrease. Similarly the acid-soluble protein pool (containing 0.7mg of protein/10^8 cells initially) decreases rapidly during development and reaches a low value of 0.1–0.2mg of protein/10^8 cells after 15h of development on Millipore filters and this value then remains constant. Neither the rate nor the extent of these decreases showed any consistent changes while the initial glycogen content of the myxamoebae was varied from 0.07 to 5.6mg of glycogen/10^8 cells.

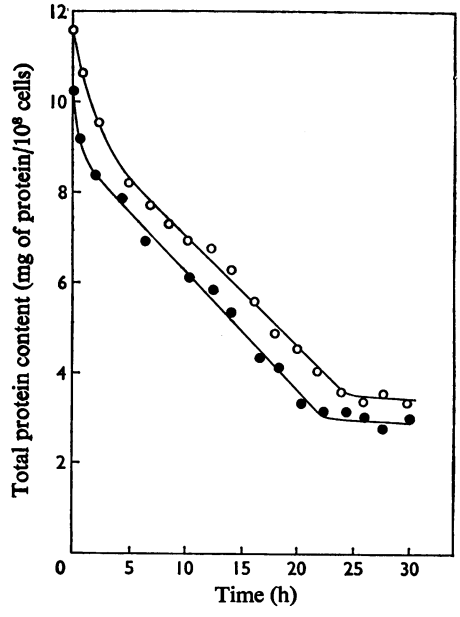


Fig. 6. *Changes in the acid-insoluble protein content of cells during the development of axenically grown myxamoebae*

Myxamoebae were grown in axenic medium and contained initially, per 10^8 cells: 0.32mg of glycogen (○); 1.24mg of glycogen (●). For details see the text.

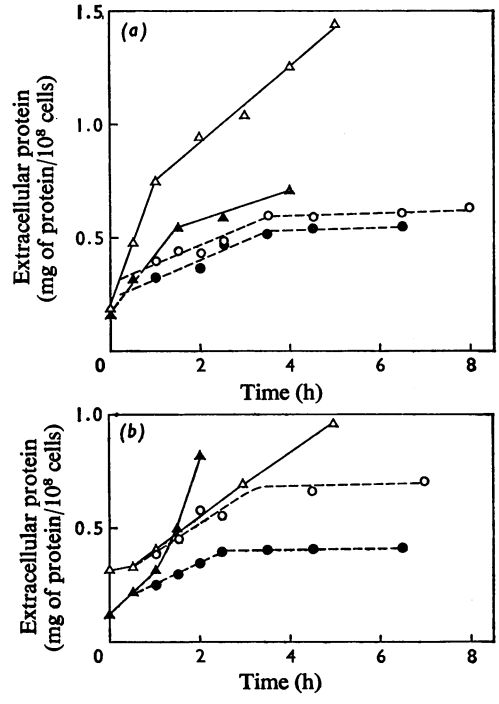


Fig. 7. *Excretion of cellular protein by axenically grown myxamoebae*

(*a*), Myxamoebae contained initially, per 10^8 cells, 0.30 mg of glycogen (△, ○); 0.28mg of glycogen (▲, ●) and were shaken in water (△, ▲) or incubated on Millipore filters (○, ●). (*b*) Myxamoebae contained initially, per 10^8 cells, 1.53mg of glycogen (△, ○) or 1.95mg of glycogen (▲, ●) and were shaken in water (△, ▲) or incubated on Millipore filters (○, ●). For details see the text.

Extracellular protein

The initial rapid loss of cellular protein (Fig. 6) may be caused either by intracellular protein utilization or by protein excretion, and so the protein present in the extracellular fluid was determined. Myxamoebae were harvested by the standard procedure and resuspended in water. Some of these cells were allowed to differentiate on Millipore membrane supports, but 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 after resuspension in water, and that this extrusion continues, although sometimes at a lower rate, for approx. 2–3h after the cells are deposited on Millipore membrane supports (Fig. 7) with a concomitant fall in intracellular Folin-positive material (Fig. 6). The absolute amount of material excreted during development is variable, but is usually less than 1.0mg of bovine serum albumin equivalents/10^8 cells. During the course of these experiments cells were washed from the Millipore filter supports and counted, which showed that less than 5% of the cells lysed while the cells were on the Millipore filter supports and therefore the protein in the extracellular buffered medium was almost certainly excreted protein. (Cells suspended in water are usually stable for at least 4h 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 3h 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.

The presence of high concentrations of myxamoebal glycogen has no effect on the extent of excretion of Folin-positive material (Fig. 7) or on the initial rapid decrease in cellular protein content (Fig. 6).

Extracellular amino acids

Attempts were made to determine the amounts of amino acids excreted during development by using

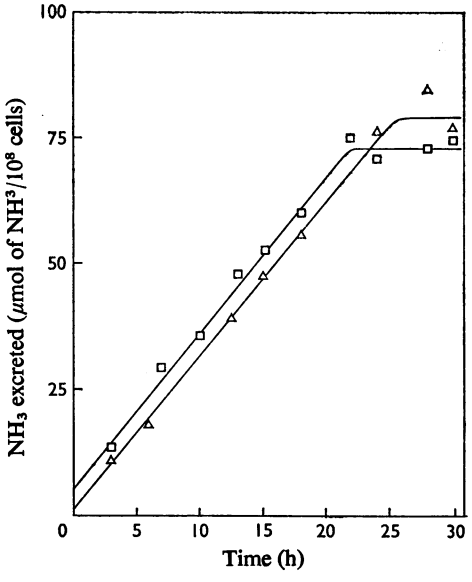


Fig. 8. *NH_3 production during the development of cells derived from axenically grown myxamoebae*

Myxamoebae were grown in axenic medium and contained initially, per 10^8 cells: 0.17mg of glycogen (□); 2.45mg of glycogen (△). For details see the text.

the ninhydrin assay (Mitchell & Moyle, 1953) and correcting for the reaction of NH_3 with ninhydrin by the glutamic dehydrogenase method of Levitzki (1971). However, the large amounts of NH_3 in the buffered salts solution prevented accurate and reproducible measurements of excreted amino acids. Thus to determine whether the presence of high concentrations of glycogen in myxamoebae decreases 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 buffer was monitored. The results showed that myxamoebae excrete similar amounts of radioactive material (equivalent to 4% of the myxamoebal protein content) during development, irrespective of cellular glycogen content.

Production of NH_3 during development

Axenically-grown cells excrete large quantities of NH_3 during development, the excretion occurring immediately after the cells are deposited on Millipore filter supports and continuing at a linear rate until culmination (Fig. 8). High concentrations of intracellular glycogen appear to have little effect on either the rate or extent of NH_3 excretion (Fig. 8).

Production of CO_2 during development

It has been shown above that myxamoebal glycogen is oxidized to CO_2 during development. Therefore to measure the production of CO_2 from protein, cells were labelled with [U-^{14}C]aspartate under conditions such that most of the label enters protein and not glycogen. Oxidation of protein to CO_2 was then measured by trapping $^{14}CO_2$ in NaOH and then determining the radioactive content of this alkali. This showed that most of the cellular protein degraded during development is oxidized to CO_2 and that the oxidation of up to 2.81mg of glycogen/10^8 cells has no significant effect on the extent of this protein oxidation (Table 6). Mass spectroscopy (Hames, 1972) was used to show that the gas trapped in the alkali was mainly CO_2.

Cellular RNA metabolism during development

Cellular RNA decays rapidly from the onset of development, according to first-order kinetics (Fig. 9), and the value of the first-order rate constant determined for myxamoebae initially containing various concentrations of glycogen indicates that the rate of cellular RNA degradation is unaffected by the quantity of myxamoebal glycogen present. Also there is a marked variability in the myxamoebal RNA content (mean 314, range 265–484μg of ribose equivalents/10^8 cells) which was not correlated with the glycogen content of the myxamoebae. White & Sussman (1961), studying bacterially-grown myxamoebae, reported a similar variability and this was explained by Hanks (1967) as being due to differences in the stage of growth of the myxamoebae used. However, in our experiments there seems 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, although we have no direct evidence for this. However, since both the myxamoebal RNA content 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, this suggests that much of the variability is due to the extremely rapid rate of RNA degradation after removal of nutrients (Fig. 9). Relatively minor differences in the time taken to harvest myxamoebae and to obtain the early cell samples would be expected to result in

Table 6. *Oxidation of [^{14}C]-labelled protein, synthesized by myxamoebae during the growth phase from [U-^{14}C]aspartate, during their subsequent differentiation*

Myxamoebae initially contained either 0.26mg of glycogen/10^8 cells (Expt. 1) or 2.81mg of glycogen/10^8 cells (Expt. 2) and were allowed to develop in closed Conway vessels as described in the Experimental section.

Sample (material and developmental time)	Total radioactivity (c.p.m./10^8 cells)		Specific radioactivity (c.p.m./μg-atom of carbon)	
	Expt. 1*	Expt. 2*	Expt. 1*	Expt 2*
Total cells				
0h	44735	17494	—	—
90h	16388	5194	—	—
Cellular glycogen				
0h	3333	1479	387	16
90h	944	240	494	98
Cellular protein				
0h	31000*	13700*	48	26
90h	6750*	2500*	44	22
CO_2				
90h	31360	13120	—	—
Pad buffer				
90h	1910	640	—	—

* These values are estimates based on: (1) the specific radioactivity of acid insoluble protein; (2) the total protein content of cell extracts, and thus do not take account of the specific radioactivity of cellular trichloroacetic acid-soluble protein; (3) the assumption that the average molecular weight of the amino acids of *D. discoideum* protein is 100.

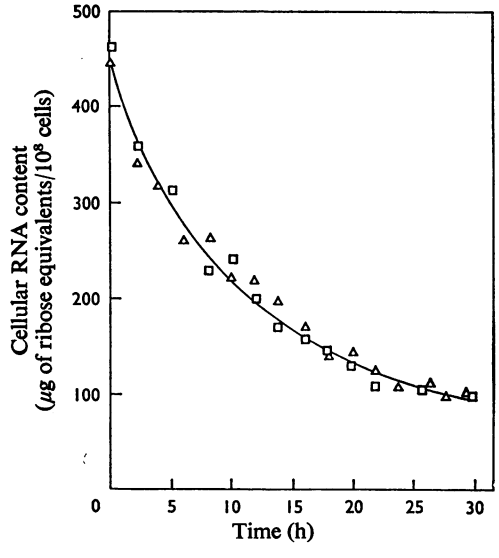


Fig. 9. *Changes in the cellular RNA content during the development of axenically grown myxamoebae*

Myxamoebae were grown in axenic medium and contained initially, per 10^8 cells: 0.094mg of glycogen (□); 1.11mg of glycogen (△). For details see the text.

fairly large differences in observed vegetative myxamoebal RNA content, and hence the amount of RNA degraded is unaffected by the oxidation of large amounts of myxamoebal glycogen (Fig. 9).

Intracellular trichloroacetic acid-soluble orcinol-positive material

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

Measurement of acid-soluble orcinol-positive material indicates that soluble pentose intermediates do not accumulate during development but rather decrease rapidly (Fig. 10). The myxamoebal pool of acid-soluble orcinol-positive material is variable in size, but during development decreases to approx. 20μg of ribose equivalents/10^8 cells at 25h developmental time irrespective of the initial pool size or myxamoebal glycogen content (Fig. 10). [It should be noted that not all of the material present in the acid-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 small amounts are extruded compared with the amount of RNA degraded, and the amount excreted does not vary significantly with changes in the myxamoebal glycogen content.

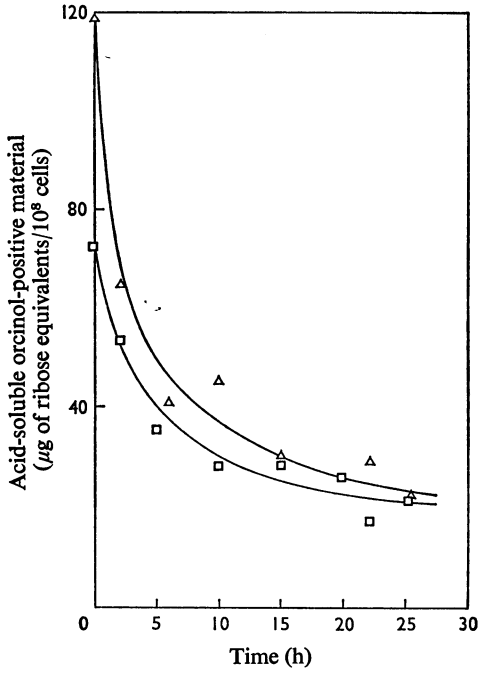


Fig. 10. *Changes in the cellular, acid-soluble, orcinol-positive material during the development of axenically grown myxamoebae*

Myxamoebae were grown in axenic medium containing initially, per 10^8 cells: 0.26mg of glycogen (□); 3.62mg of glycogen (△). For details see the text.

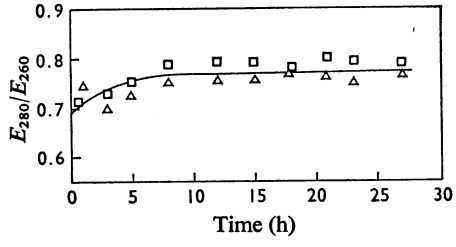


Fig. 11. *Changes in amount of extracellular u.v-absorbing material during the development of axenically grown myxamoebae*

Myxamoebae were grown in axenic medium containing initially, per 10^8 cells: 0.29mg of glycogen (□); 6.77mg of glycogen (△). A value of 1.0 was obtained for the E_{260} of both sets of cells at the earliest time of sampling.

Extracellular u.v.-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. Fig. 11 shows that axenically-grown cells excrete u.v.-absorbing compounds, but that the excretion follows the same time-course as excretion of Folin-positive material (see Fig. 6). Moreover, the E_{260}/E_{280} ratio increases only slightly or not at all (depending on the experiment) during the first 3h of development and then remains constant (Fig. 11). These data are incompatible with RNA degradation, retention of RNA bases in the cells and their subsequent excretion after several hours of development.

Discussion

The differentiation phase of the life cycle of the cellular slime moulds is initiated when the myxamoebae stop growing and dividing. Since this is usually achieved by removing the myxamoebae from a utilizable source of nutrients, the assumption has been made that 'starvation' acts as a trigger, causing the cessation of growth and the initiation of the aggregation process. At the biochemical level differentiation is thus seen as a response to a diminished energy supply reflected, for example, in an increase in the inorganic phosphate pool (Wright, 1973). However, we have shown that it is possible to alter the glycogen content of the myxamoebae by 100-fold and that the myxamoebae follow the same developmental pathway in the same time (with the exception of relatively minor quantitative variations, Garrod & Ashworth, 1972) whatever their initial glycogen content (Table 1). Since the myxamoebal glycogen can be and is metabolized (Fig. 3), it is clear that the cells cannot be starving in the sense that they lack a readily utilizable oxidizable carbon source. The regulatory mechanism(s) which initiate development are unlikely therefore to be directly connected with energy supply or availability *per se*. It has been further argued that the marked decrease in cellular protein and RNA content that accompanies differentiation (White & Sussman, 1961) is due to the fact that differentiating cells need to mobilize their macromolecules for energy provision since they lack other energy reserves. However, since the provision of an alternative source of utilizable energy (glycogen) has no effect on the rate or the extent of breakdown of cellular protein or RNA (Figs. 6 and 9), it is clear that this argument is invalid and we conclude that protein and RNA molecules are degraded for reasons other than the necessity to provide oxidizable substrates for energy provision.

Previous work (Sussman & Sussman, 1969) on the overall metabolism of differentiating myxamoebae has used myxamoebae grown on bacteria as source of nutrients. These studies by Sussman & Sussman (1969) have shown that there is little net change in carbohydrate content of the cells during

differentiation, but that there is a considerable and extensive qualitative change in the nature of the carbohydrates present in cells at different developmental stages. These facts have been taken to imply that gluconeogenesis (Cleland & Coe, 1969) and glycolysis are negligible, and ambitious attempts to simulate the resulting metabolic pathways involved in conversion of glycogen into cellulose, trehalose and mucopolysaccharide (which form a closed network) have been made (Wright, 1973). However, the present paper shows that differentiation can proceed, following the same morphogenetic progression and at the same rate whether the differentiating cells are carrying out net gluconeogenesis (Fig. 2) or glycogenolysis (Fig. 3). The observations made by White & Sussman (1961) must be taken therefore to reflect biochemical events relevant not to the differentiation processes themselves, but merely to that particular set which occur in myxamoebae containing approx. 0.5mg of glycogen/10^8 cells initially. Thus whereas simulation studies of the carbohydrate metabolism of such cells might reveal 'critical variables' (Wright, 1973) relevant to the particular metabolic conditions of bacterially grown cells, these variables cannot also be critical for the developmental processes since it is clear that these can occur unchanged despite dramatic alterations in the pattern of carbohydrate metabolism.

Our studies have also revealed complexities in the metabolism of carbohydrates in *D. discoideum* which have not been reported before. Thus even in myxamoebae which contain little glycogen initially and which undertake considerable net gluconeogenesis in the course of differentiation, there is an initial period in which there is a net decrease in cellular carbohydrate (Fig. 2). White & Sussman (1961) reported a similar change in their studies of bacterially grown myxamoebae, but they ascribed this to the degradation of residual bacterial glycogen present in food vacuoles of the myxamoebae. This explanation cannot account for our findings which suggest that there is an initial 2–3 h period when there is a very rapid mobilization of carbohydrate reserves. This distinction between the initial and later phases can also be seen in myxamoebae which contain a large amount of glycogen (Fig. 3) and which never show net gluconeogenesis. The enzymes involved in catalysing the glycogenolysis shown in Fig. 3 seem to be of the amylase type (Fig. 4) since differentiating cells do not contain significant glycogen phosphorylase activity until the latter stages of differentiation (Fig. 5). It is clear, however, that there is no induction of amylases by glycogen since cells have the same initial specific activity of amylase which changes in a characteristic pattern during development despite considerable initial differences in glycogen content (Fig. 4). Comparison of Figs. 3 and 5 also reveals an apparently paradoxical situation for the enzyme glycogen phosphorylase is not, apparently, induced until a time (14–16h) when most of the myxamoebal glycogen has been metabolized, and thus when the greater part of the enzyme's substrate has apparently disappeared. Further, in the case of this enzyme, there is a clear dependence of the amount of enzyme synthesized by the differentiating cells on the amount of glycogen present in the myxamoebae from which they were derived. It is conceivable that phosphorylase might act as a synthetic enzyme in these cells but since they have, at all times of development, considerable glycogen synthetase activity (Hames *et al.*, 1972) and since there is no precedent for such a suggestion, we postulate that there exist two separate pools of glycogen, one present before the 14h period derived largely from the myxamoebae and the other, present after this time, synthesized, in part, *de novo*. We have previously shown that the rate of glycogen synthesis reaches a peak in cells derived from myxamoebae containing 2.7mg of glycogen/10^8 cells initially at 10–14h (Hames *et al.*, 1972), and in the present paper we have shown (Table 4) that even in cells derived from myxamoebae containing a very high glycogen content initially there is a significant contribution to the carbon atoms of the end-product saccharides (cellulose, trehalose and glucose) by gluconeogenic precursors. Whether these postulated two pools of glycogen can be physically, as opposed to functionally, distinct is not clear. There is no apparent separation of the myxamoebal glycogen from the cytoplasm (Plate 1) and since it is possible for the amount of glycogen present to affect the amount of end-product saccharides synthesized (Table 3; Hames & Ashworth, 1974), the postulated pools must interact to some extent. If the existence of two interacting pools of glycogen is accepted, one derived largely from the myxamoebae and metabolized to CO_2 and water after amylytic attack, and the other derived in part from gluconeogenesis and giving rise to the end-product saccharides after phosphorylytic attack, then the attempts to model the biochemical changes along the lines suggested by Wright *et al.* (1968) may still be possible provided that it is only the second of these pools that is considered.

Few studies have been reported on the mechanism(s) of protein degradation during development in *D. discoideum*. There is no apparent induction of lysosomal proteases during development (Wiener & Ashworth, 1970), although increases have been reported in a number of enzymes concerned with amino acid oxidation (Pong & Loomis, 1973; Firtel & Brackenburg, 1972). The metabolic significance of these increases remains unclear, since there is no apparent change in the rate of NH_3 production (Fig. 8) at any time during development. However, it is clear that the major

portion of the degraded protein is oxidized to CO_2 and water (Table 6) and the provision of an alternative metabolizable energy source such as glycogen has little or no effect on the extent to which protein is oxidized to CO_2 (Table 6) and NH_3 (Fig. 8). Therefore the massive net loss in protein that occurs during development occurs for reasons other than the necessity of providing oxidizable substrates for energy provision, and thus the mechanism of protein degradation and its control emerges as a key biochemical problem during development.

Similar conclusions can be drawn from our studies on the pattern of RNA degradation which is also unaffected by the presence of a considerable excess of glycogen in the cell (Figs. 9, 10 and 11). Recently much attention has been focused on the appearance of new, or enhanced, concentrations of specific proteins and RNA molecules during the development of *D. discoideum*; the present paper suggests that of equal, if not more, importance is the disappearance of such materials.

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