

ATP, TREHALOSE, GLUCOSE, AND AMMONIUM ION
LEVELS IN THE TWO CELL TYPES OF
DICTYOSTELIUM DISCOIDEUM

by

Jeanne Burrowbridge Wilson

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APPROVED:

C. L. Rutherford, Chairman

A. A. Yousten

D. A. Stetler

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 Dictyostelium discoideum: A Model System

In the study of cell differentiation, Dictyostelium discoideum offers several advantages over model systems used in the past (Bonner, 1971; Gregg, 1966). Among these advantages are the following. Metabolism in D. discoideum becomes endogenous when the bacterial food supply is insufficient for the support of vegetative growth. The process of growth and division are distinctly separated from the process of cell differentiation. During the life cycle one cell type differentiates into two cell types, rather than many types of cells as in other developmental models. And finally, the sequence of development in this system can be identified by discrete morphological steps.

The stages in the life cycle of D. discoideum are illustrated in Figure 1. The germination of a single spore yields a single myxamoeba which can ingest bacteria and divide by mitosis. When the myxamoebae have depleted the food supply the vegetative cycle ceases and cell differentiation commences. Approximately 7 hours (h) after the onset of starvation the amoebae begin to form multicellular aggregates. An aggregate is formed as several hundred to several thousand amoebae stream together in response to a chemotactic agent, "acrasin", currently thought to be 3',5' cyclic adenosine monophosphate (Bonner et al., 1969). When the aggregate is complete, about 12h after development begins, it falls over onto its side and commences

a period of migration. During this pseudoplasmodium, or slug, stage differences between cell types first appear. Biochemical (Gregg, Hackney, Krivanek, 1954; Rutherford, 1976), histochemical (Krivanek and Krivanek, 1958; Bonner, 1971; Farnsworth and Loomis, 1974), and morphological (Raper, 1940; Hohl and Hamamoto, 1969; Gregg and Badman, 1970) experiments have indicated that the anterior one-third of the pseudoplasmodium contains presumptive stalk cells while the posterior two-thirds are comprised of prespore cells. Also during this stage, the cells secrete a mucopolysaccharide slime sheath which is deposited behind the pseudoplasmodium as it migrates. At about 20h into the developmental life cycle, the pseudoplasmodium approaches radial symmetry in preparation for the formation of a mature fruiting body. During the 4-5h culmination process, prestalk cells migrate up toward the tip of the organism and down into a centrally located cellulose sheath. The prestalk region during culmination has been shown to be highly active biochemically (Krivanek and Krivanek, 1958; Jefferson and Rutherford, 1976). It has been suggested that the apical region of the prestalk area may organize and direct cell migration throughout the entire organism (Raper, 1940; Rubin and Robertson, 1975; Farnsworth, 1973). The stalk cells within the sheath become highly vacuolated and develop rigid cell walls (Raper and Fennell, 1952), while cells within the prespore mass begin to form the initial layers of a strong spore coat (Hohl and Hamamoto, 1969). The migration of prestalk cells into the sheath and the construction of a rigid cellulose stalk pull the prespore mass up off of the substrate toward

the top of the forming stalk (Bonner, 1971). The mature fruiting body, or sorocarp, consists of a cellulose stalk (2 mm high) which supports a spore mass at its apex. The sorocarp is complete approximately 24h after the beginning of development.

The process of development, briefly described above, involves both temporal and spatial differentiation. These types of differentiation require biological decisions concerning the formation of patterns during development (Wolpert, 1969; Bonner, 1971; Babloyantz and Hiernaux, 1974). Pattern formation in the development of D. discoideum is denoted by the strict proportionality of prespore and prestalk cells both during the early stages of the life cycle and in the completed sorocarp. These patterns are probably formed through cellular interactions (perhaps gradients) which can relate positional information (Wolpert, 1969; Babloyantz and Hiernaux, 1974). Cellular interaction is indicated by the ability of D. discoideum to form a normal sorocarp from isolated sections of prespore tissue (Raper, 1940). The interaction of cells under these conditions results in the reapportioning of the cell types. Other examples of cellular communication in this system are the sensitivity of aggregating amoebae to acrasin and the morphological and functional polarity of pseudoplasmodia (Gregg, 1966). Elucidation of the mechanisms involved in these cellular interactions, be they gradients, thresholds, or phase shifts (Goodwin and Cohen, 1969), depends on the capability for the study of cell-specific events. A knowledge of events occurring in a specific cell type, at a specific time and place during development

could reveal some of the regulatory mechanisms of cell differentiation. The investigation of cell-specific events in D. discoideum has been made possible by the adaptation of an ultramicrochemical technique developed by O. H. Lowry (Lowry and Passonneau, 1972).

1.2 Energy Metabolism in D. discoideum: ATP, NH_4^+ , Trehalose and Glucose

This thesis reports cell specific levels of ATP, ammonium ion (NH_4^+), trehalose, and glucose in D. discoideum. The probable significance of these substances in the development of this organism is reviewed below.

Farnsworth and Loomis (1974, 1975) have suggested that the increasing thickness of the slime sheath from the anterior to the posterior end of pseudoplasmodia may cause differential diffusion between cells thus creating gradients of certain metabolites (see also Babloyantz and Hiernaux, 1974; Loomis, 1972; Watts and Treffry, 1975). These gradients may contribute to the regulation of spatial patterns of differentiation. Both ATP and NH_4^+ are metabolic effectors in other systems (Atkinson, 1966; Otto et al., 1974; Eckert et al., 1975; Hato et al., 1976), and both may be diffusible molecules whose gradients effect cell-specific differentiation in D. discoideum (Loomis, 1972). A gradient of ATP in the pseudoplasmodium would yield a differential energy source which could influence pattern formation and the regulation of developmental metabolism. This influence might distinguish between cells at various positions along the length of a pseudoplasmodium (prestalk versus prespore cells) or between cells

on the periphery of the organism and those deeply embedded in the cell mass. Ammonium ion, a metabolic inhibitor in other biological systems (Tager et al., 1975), could cause cell-specific differentiation if cells were exposed to a gradient of this effector. High concentrations of NH_4^+ , influencing metabolism in only one region of D. discoideum as a result of differential diffusion, might modulate the accumulation of endproducts which are specific to a distinct cell type. Thus, the thickness of the slime sheath at the posterior end of the pseudoplasmodium may cause accumulation of NH_4^+ in that region and, thereby, contribute to spore-cell differentiation.

ATP levels and the accumulation of NH_4^+ are important later in development also. As one factor in the computation of energy charge (Atkinson, 1968), ATP can indicate the metabolic condition of an organism (Gadkari and Stolp, 1975). This condition is reflected by the metabolic balance between ATP-utilizing reactions and ATP-regenerating reactions (Atkinson, 1968). D. discoideum is a developmental system geared toward cell differentiation and biosynthesis in an endogenous metabolism. In this system the production of carbohydrate endproducts by newly synthesized proteins for cell differentiation is balanced with the degradation of unneeded proteins, lipids, and nucleic acids (Gustafson and Wright, 1972; Loomis, 1975). By examining cell-specific ATP levels one could determine when, during development, or in which cell type the metabolic balance between degradation and biosynthesis begins to shift (Chulavatnatol and Atkinson, 1973). The accumulation of NH_4^+ can be interpreted as an indication of

protein degradation, an energy source during development of D. discoideum (Gregg, Hackney, and Krivanek, 1954; Wright and Anderson, 1960). Therefore variation in the cell-specific pattern of NH_4^+ accumulation could reveal differential protein degradation and energy requirements.

Cotter and Raper (1970) have postulated that the disaccharide, trehalose, is an energy source for the emergence of amoebae during spore germination in D. discoideum. They also suggested that glucose was an energy source during earlier phases of spore germination. Therefore, one might expect trehalose and glucose to be found only in spores. However, Rutherford and Jefferson (1976) showed that trehalose accumulated in both cell types during development, but was retained only in mature spores. This finding correlates well with their observation of very high trehalase activity in stalk and prestalk cells (Jefferson and Rutherford, 1976a). To support these results, the cell-specific accumulation of both trehalose and glucose, the product of its degradation, were also investigated during the research reported here.

2. MATERIAL AND METHODS

2.1 Chemicals

All reagents were purchased from Sigma Chemical Company (St. Louis, Missouri) and stored either frozen or under refrigeration. Agar, yeast extract, and peptone were obtained from Difco Laboratories (Detroit, Michigan).

2.2 Growth and Preparation of D. discoideum

Spores from D. discoideum were mixed with exponentially growing broth cultures of Escherichia coli. This mixture was the inoculum for pans of nutrient agar on which the spores germinated. After most of the bacteria had been consumed, but aggregation had not yet begun, the amoebae were harvested and plated on filter pads supported on non-nutrient agar medium. When the desired stage of development was reached, the filters were removed, quickly frozen, and the lyophilized slime molds were stored under vacuum at -20°C .

Escherichia coli were inoculated into 10 ml of nutrient broth (containing 1.0g yeast extract, 10g dextrose, 10g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1.0 liter of 0.01 M potassium phosphate buffer, pH 6.5) and incubated at 23°C for 24h. Spores from D. discoideum NC-4 were added to the turbid broth. This suspension was used to inoculate stainless steel pans (16 x 11 x 1 inches, Volrath Co., Sheboygan, Wisconsin) containing 500 ml of complex solid media. This media contained 1.0g yeast extract, 10g dextrose, 10g peptone, 20g agar and 1.0g

MgSO₄·7H₂O in 1.0 liter of 0.01 M potassium phosphate buffer (pH 6.5). After being inoculated with 2.5 ml of the broth suspension, the cultures were covered and maintained at 23°C for 48 hr. During this incubation most of the bacteria were consumed.

The amoebae were harvested by the method of Liddel and Wright (1961) and allowed to differentiate on non-nutrient media. After being washed from the agar surface with cold glass-distilled water, the amoebae were pelleted in a refrigerated centrifuge (Sorvall SS-3 Automatic) at 1000 x g. This pellet was resuspended in cold glass-distilled water and centrifuged at 750 x g to wash the amoebae free of any remaining bacteria. A final suspension of amoebae in cold glass-distilled water served as the inoculum for petri dishes (15 mm x 140 mm dia) which contained discs of Whatman No. 50 filter paper on non-nutrient agar. The non-nutrient media consisted of 20g agar and 1.0 mM EDTA in 1.0 liter of 0.01 M potassium phosphate buffer (pH 6.5). After spreading each filter with 1.3 ml of the amoebae suspension, the petri dishes were covered and maintained at 23°C while differentiation progressed.

At the desired stage of development, the filter was transferred to dry ice which froze the slime molds within one second. When all stages had been collected in this manner, the filters were placed in a Freeze Dry Apparatus (Model 10-800, Virtis Research Equipment, Gardiner, New York). After lyophilization for 36h at -40°C and less than 10 microns Hg, the filters were cut into 2 x 4 cm pieces and stored in screw cap vials (No. 10-159-10, Virtis Co., Gardiner, N.Y.)

under vacuum at -20°C . On the day of an assay, the vacuum vial was allowed to reach room temperature before the vacuum was released and individual slime molds were removed.

2.3 Description of the Microtechnique

The ultra-microchemical technique involves the biochemical assay of enzyme substrates, products, or specific activities in microgram quantities of tissue. It was applied, in these investigations, to small ($15.0-0.02 \mu\text{g}$) sections of slime mold tissue. The size of these samples demanded special methods for handling and assay. Since final results were expressed in terms of dry weight, each piece assayed (either intact individual or section thereof) had to be accurately weighed. This was accomplished with a quartz-fiber balance that had been calibrated with crystals of para-nitrophenol (Lowry and Passonneau, 1972). The individual to be analyzed was dissected with a microscalpel (if sections were desired) and transported to and from the quartz-fiber balance on a hairtip as described by Harris (1975). After being weighed, the sample tissue was assayed. The initial reactions occurred under mineral oil (Lowry and Passonneau, 1972) in a well (4 mm x 4 mm dia.) drilled into a block of teflon (20 x 120 x 5 mm). The weighed tissue was transferred into a small volume of reaction mixture in the bottom of the well. This reaction mixture was covered with a solution of hexadecane and mineral oil (3:7 v/v) to prevent evaporation during ensuing manipulations. Subsequent

reaction mixtures were added to the volume in the oil well with a hand-made constriction pipette.

Perhaps the most integral aspect of the microtechnique involves the amplification of the native fluorescence of pyridine nucleotides. These fluorescent properties permitted the quantitative amplification of substrates or enzymes in the tiny pieces of tissue to levels which were within the range of fluorometric measurement. The two methods of achieving amplification were either (1) treatment with strong alkaline (6N NaOH) or (2) enzymatic cycling by a coupled system of dehydrogenases (Lowry and Passonneau, 1972). The level of substrate, product, or enzyme activity to be assayed determined the procedure used for amplification. At levels that ranged from 10^{-9} mole to 10^{-10} mole NaOH treatment was used for amplification, whereas levels between 10^{-11} mole and 10^{-15} mole required enzymatic cycling. This amplified fluorescence was measured on a fluorometer (Model A-4, Farrand Optical Co., New York, N.Y.) equipped with a Corning No. 5840 filter for incident light and filters No. 3387 and No. 3408 for emitted light. Enzyme substrate levels were reported as mmol/kg dry weight.

Because the levels of metabolites in the tissue sections were low (10^{-15} mole) special precautions were necessary to insure that the assays were valid. The pH changes resulting from the addition of microliter volumes of subsequent reaction mixtures could not be measured. Therefore, these changes were noted in larger volumes to assure that the optimal pH was maintained during enzymatic reactions. The enzymes used in the cycling procedures were Norite treated to

remove any nucleotide contamination, as described by Harris (1975), with the following modification for glucose-6-phosphate dehydrogenase (G6PDH). This enzyme was purchased as a lyophilized powder and reconstituted with Tris-HCl buffer 0.1 M (pH 8.1) containing 0.1% bovine serum albumin (BSA). The Norite pellet was resuspended in this enzyme solution prior to incubation and centrifugation. Glutamate dehydrogenase (GDH) in glycerol was also Norite treated (Harris, 1975) for use in the cycling procedures. An additional volume of GDH in glycerol was dialyzed at 4°C for 48h against potassium phosphate buffer 0.01 M (pH 7.4) containing 0.2 M NaCl to remove any contaminating ammonium ions (Tabor, 1970). This dialyzed GDH was used in the first reaction in the assay for ammonium ion (Figure 2). The trehalase required by the assay for trehalose was centrifuged at 100 x g in membrane cones (Centriflo type CF 25, Amicon Corp., Lexington, Mass.) to remove glucose contamination. These treatments and procedures resulted in minimal blank fluorescence and permitted the collection of reliable data.

2.4 Assay Procedures

2.4.1 ATP

Both intact individuals and sections of individuals were assayed for ATP content by the enzymatic reactions listed in Figure 3. The nucleotide levels produced during the initial tissue reactions were amplified by enzymatic cycling. The major product of the cycling reactions, 6-phosphogluconate, was converted to ribulose-5-phosphate

and the concomitant production of NADPH was measured on the fluorometer.

Single, whole individuals from stages throughout the developmental cycle were assayed to determine ATP content during development. Each weighed individual was placed into 0.563 μ l of 0.02 N NaOH in the bottom of an oil well. After covering each droplet with oil, the teflon rack was heated at 90°C for 15 min to destroy any active tissue enzymes. When the rack had returned to room temperature, 0.794 μ l of reaction mixture (0.05 M Tris-HCl buffer (pH 8.1), 1.57 mM MgCl₂, 0.76 mM dithiothreitol (DTT), 0.49 mM glucose, 0.49 mM NADP⁺, 1.22 U/ml G6PDH, and 1.73 U/ml hexokinase) was added to each well. The rack was floated on a 37°C water bath for 60 min. During this incubation the ATP present in the sample tissue was consumed in the production of 6-phosphogluconate and an equimolar amount of NADPH (Figure 3). To destroy any excess NADP⁺, 0.794 μ l of 0.3 N NaOH was added to each well and the rack was incubated at 60°C for 10 min. The rack was allowed to cool to room temperature again before 10.6 μ l of cycling reaction mixture was added to each well. This reaction mixture contained 0.1 M Tris-HCl buffer (pH 8.1), 7.81 mM α -ketoglutarate (α KG), 3.88 mM glucose-6-phosphate (G6P), 0.18 mM ADP, 32 mM ammonium acetate, 0.03% BSA, 0.3 U/ml G6PDH, and 1.54 U/ml GDH. During the incubation at 37°C for 60 min approximately two hundred amplifying cycles of the reactions shown in Figure 3 occurred. Then the rack was heated at 90°C for 15 min to halt the cycling process by denaturing the enzymes. The entire reaction volume of each well was

transferred to 1 ml of 6-phosphogluconate (6PG) reaction mixture in 3 ml fluoremeter tubes. This reaction mixture contained 0.1 M Tris-HCl buffer (pH 8.3), 0.14 mM EDTA, 30 mM ammonium acetate, 10 mM MgCl_2 , 0.03 mM NADP^+ , and 0.072 U/ml 6-phosphogluconate dehydrogenase. These tubes were incubated at 37°C for 45 min. After this final conversion to NADPH (Figure 3), the amplified fluorescence was measured on a fluorometer as described in Section 2.2. Standards containing 1.93×10^{-11} , 3.86×10^{-11} , and 5.79×10^{-11} mole of ATP were included in all assays.

Sections of individuals were assayed to determine ATP content within the two cell types. Each section was placed into 0.780 μl of 0.02 N NaOH in the bottom of an oil well. After each droplet was covered with oil, the rack was heated at 90°C for 15 min to destroy active tissue enzymes. To each cooled well 0.253 μl of ATP reaction mixture was added. When assaying sections of individuals, this reaction mixture contained 0.5 M Tris-HCl buffer (pH 8.1), 4.54 mM MgCl_2 , 1.93 mM DTT, 0.49 mM glucose, 0.09 mM NADP^+ , 0.08% BSA, 0.35 U/ml G6PDH, and 1.25 U/ml hexokinase. It was necessary to compensate for the large volume of NaOH to which this reaction mixture was added by increasing the molarity of the buffer. This permitted maintenance of the optimum pH for the enzymatic reactions which produced NADPH. The inclusion of BSA in this reaction mixture prevented the surface denaturation of G6PDH and hexokinase in the tiny reaction volume. The amount of NADP^+ was reduced from that used in the assay of whole individuals. This retained levels of NADP^+ in excess of ATP levels expected in

small sections, but insured complete destruction of the excess NADP^+ by heating in NaOH. After incubating the ATP reaction mixture in the wells at 37°C for 60 min, $0.780\ \mu\text{l}$ of $0.3\ \text{N}$ NaOH was added to each well and the wells were incubated at 60°C for 30 min to destroy excess NADP^+ . After cooling, $8.06\ \mu\text{l}$ of cycling reaction mixture was added to each well. This reaction mixture was the same as that used for whole individuals except that G6PDH and GDH were increased to yield approximately ten thousandfold amplification. The ratio of these two enzymes was also adjusted to achieve optimum cycling (Figure 4). After incubation the oil-well rack was heated at 90°C for 15 min. The entire reaction volume of each well was transferred to 1 ml of 6PG reaction mixture in 3 ml fluorometer tubes. This reaction mixture was identical to that used for the assay of whole individuals. These tubes were incubated at 37°C for 45 min to allow the final production of NADPH by the reaction noted in Figure 3. The amplified fluorescence was measured on a Farrand fluorometer (Section 2.2). Standards containing 3.6×10^{-13} and 7.2×10^{-13} mole of ATP were carried through all steps of the tissue assays.

To confirm that spores were broken by the assay procedures described above, extracts were prepared in which the spores were ruptured by passage through a French pressure cell prior to being assayed for ATP. Extracts from spore cells (36h into development) were prepared by dissecting the spore mass from approximately 40 freeze-dried individuals. The tissue was combined, weighed (Automatic Electrobalance Model 4700, Cahn Instruments, Cerritos, Ca.), and then

dissolved in 3 ml of distilled water. After heating the extract at 100°C for 4 min, one half of this volume was passed through a French pressure cell (Carver Laboratory Press Model C; Fred S. Carver, Inc., Menomonee Falls, Wis.) under 20,000 psi at 4°C. The remaining extract was used as a control. Samples (0.780 μ l) from both the pressed and the unpressed extracts were assayed for ATP content using the same reaction mixtures and volumes as those employed in the assay of sections of individuals.

2.4.2 Trehalose and Glucose

Sections of individual slime molds were assayed to determine cell-specific contents of trehalose and glucose. These assays used the enzyme reactions noted in Figure 5. For the assay of glucose each weighed sample was placed in 0.794 μ l of 0.02 N HCl in an oil well. After all samples had been placed into wells and covered with oil, the racks were heated at 90°C for 15 min to inactivate tissue enzymes. It was found that the addition of samples to 0.02 N HCl instead of 0.02 N NaOH allowed more accurate recovery of the glucose standards in this assay (Rutherford and Jefferson, 1976). When the wells had cooled to room temperature, 0.253 μ l of glucose reaction mixture was added to each well. This reaction mixture contained 0.68 mM ATP, 4.54 mM MgCl₂, 1.93 mM DTT, 0.14 mM NADP⁺, 0.08% BSA, 0.35 U/ml G6PDH, and 1.25 U/ml hexokinase in 0.5 M Tris-HCl buffer (pH 8.1). The wells were incubated at 37°C for 60 min while glucose was converted to 6-phosphogluconate and produced equimolar amounts of

NADPH. To destroy excess NADP^+ 0.794 μl of 0.3 N NaOH was added to each well and the rack was incubated at 60°C for 30 min. After cooling to room temperature, 8.06 μl of cycling reaction mixture was added to each well. This reaction mixture was the same as that described in Section 2.4.1 except that the cycling enzymes were increased to yield approximately a five thousandfold amplification. After cycling, the rack of wells was heated at 90°C for 15 min to destroy the enzymes. The entire reaction volume of each well was removed to 3 ml fluorometer tubes containing 1 ml of 6PG reaction mixture. This reaction mixture was identical to that described in Section 2.4.1. After incubation at 37°C for 45 min to allow the production of NADPH the amplified fluorescence in each tube was measured as described in Section 2.2.

When assaying for trehalose, the following modification was made in the glucose protocol. Trehalase (a gift from Dr. Alfred S. Sussman) was added to the reaction mixture in quantities sufficient to produce 4×10^{-10} mole glucose in 60 min at 23°C . Because the assay for trehalose also measured glucose, trehalose levels were determined by subtracting endogenous glucose levels. Both glucose and trehalose standards containing 0.75×10^{-12} and 1.5×10^{-12} mole of glucose units were carried through each assay.

2.4.3 Ammonium Ion

To determine the level of NH_4^+ in tissue and the possible significance of blanks, the assay was first attempted using an extract

derived from individuals at the pseudoplasmodium stage of development. This extract was prepared by the addition of 4.5 mg of lyophilized slugs (about 500 individuals) to 269 μ l of distilled water. After heating at 100°C for 4 min the extract was kept frozen at -20°C. The reaction employed in the assay of extract is noted in Figure 2 (Tissue Reaction). However, NaOH treatment was used in the amplification step. A sample of extract (3-10 μ g dry weight) was added to 53.0 μ l of reaction mixture containing 0.16 mM NADPH, 1.42 mM α KG and 17.7 U/ml GDH in 0.5 M Tris-HCl buffer (pH 8.3). The higher level of NADPH used in the reaction mixture was necessary to obtain a significant increase in fluorescence over the high blanks which were encountered. The tubes containing the reaction volume were incubated for 90 min at 37°C, sufficient time for the reaction to be completed. After this incubation, 35.5 μ l of 5.8 N HCl was added to each tube to destroy unreacted NADPH. To amplify the NADP⁺ which had been produced, 100 μ l of 10 N NaOH was added, each tube was stirred well, and the tubes were incubated at 60°C for 20 min. Before measuring the amplified fluorescence of NADP⁺ on the fluorometer, 1 ml of distilled water was added and each tube was stirred again. Efforts to minimize the high blank included dialyzing the GDH (Section 2.2) and degasing the other reagents. The source of NH₄⁺ or NADP⁺ contamination was investigated by varying the concentrations of the reaction mixture components. Only the dialysis of GDH was successful in lowering the blank.

When tissue sections were assayed some modifications of the procedure were necessary. In an effort to retain ammonium ions in the tissue, samples were added to acid rather than base. The addition of samples to base would have resulted in the conversion of NH_4^+ to NH_3 , therefore yielding incorrectly low values for NH_4^+ levels. The high blanks required that relatively large tissue sections (0.5 μg) be assayed. Each weighed sample was placed into 0.361 μl of 0.02 N HCl in an oil well and covered with oil. The rack of wells was then heated at 80°C for 20 min to destroy active tissue enzymes. When the rack had cooled, 0.283 μl of the initial reaction mixture was added to each well. This mixture contained 3.4 mM αKG , 0.02% BSA, 2.5 mM NADPH, and 7.5 U/ml GDH in 0.2 M Tris-HCl buffer (pH 8.5). The wells were incubated for 60 min at 37°C to permit the production of NADP^+ by the tissue reaction (Figure 2). When this incubation was completed, 0.361 μl of 0.5 N HCl was added to each well to destroy excess NADPH. After adding acid to the wells, the entire reaction volume of each well was transferred to 3 ml fluorometer tubes containing 48.5 μl of cycling reaction mixture. This cycling mixture consisted of 7.81 mM αKG , 11.8 mM G6P, 0.18 mM ADP, 32 mM ammonium acetate, 0.03% BSA, and levels of G6PDH and GDH which yielded three hundredfold amplification in the 0.1 M Tris-HCl buffer (pH 8.1). The G6P concentration in this reaction mixture was increased from levels used in previous assays to insure that an excess of this substrate was present. All tubes were heated at 100°C for 4 min to halt the cycling reactions. When the tubes had cooled, 1 ml of 6PG reaction

mixture was added to each tube. This reaction mixture was the same as that noted in Section 2.3.1 except that NADP^+ was increased to 0.19 mM. The tubes were stirred and incubated at 37°C for 45 min before the amplified fluorescence of NADPH was measured (Section 2.2). Standards of 7.6×10^{-11} mole and 15×10^{-11} mole of ammonium acetate were included in each assay.

3. RESULTS

3.1 ATP

Prior to the assay of ATP in tissues, several controls were conducted in order to demonstrate the validity of the technique. To insure that the assay measured only the triphosphate adenosine nucleotide, an experiment was performed using equal amounts (0.25 pmol) of ATP, ADP, and AMP (Table 1). Three sets of wells containing (1) ATP alone, (2) ATP and ADP, (3) ATP, ADP, and AMP were assayed as described for whole individuals (Section 2.4.1). The results of this assay indicated no additive effect due to the presence of ADP or AMP in the wells. Therefore, it was concluded that only the triphosphate adenosine nucleotide level was measured. Another control assay determined the contribution of endogenous G6P to the ATP levels detected by the assay. Because both metabolites are measured by the complete reaction mixture, the amount of G6P present in sections of mature spore and stalk tissue was determined by omitting hexokinase in the initial reaction mixture (Figure 3). G6P was an insignificant part of the ATP levels in 24h sorocarps. With each tissue experiment, known concentrations of ATP, which encompassed the expected range of tissue values, were assayed. These standards were used to: insure the linearity of the reaction within that tissue range; normalize day-to-day experimental variation; indicate the reproducibility of data within an experiment; serve as the basis for calculation of concentrations.

ATP changes during differentiation were determined in five experiments which assayed intact individuals from several stages of development (assayed as described in Section 2.4.1). These stage studies revealed that ATP dropped from approximately 7 mmol/kg at aggregation to 2 mmol/kg in 24h sorocarps. This decrease appeared to occur at the culmination stage (20h) with ATP levels before that time remaining constant. However, these stage studies reflected only average ATP levels for intact individuals, and could not disclose variation in ATP levels at different locations within an individual.

To obtain cell-specific ATP data, sections of individuals from several stages of development were assayed. At aggregation ATP levels did not vary significantly among sections from different locations (Figure 6). Cells in a streaming arm of the aggregate contained 8.3-11.0 mmol/kg, while ATP in amoebae which had already entered the central portion of the aggregate ranged from 10.6-12.5 mmol/kg.

During the pseudoplasmodium stage, when morphological differences between the cell types become apparent (Bonner, 1971), cell-specific ATP levels were determined by assaying sections from five individuals (Table 2). In two of the three individuals in which both cell types were investigated, no measurable differences were detected in the ATP content of prestalk and prespore cells. However, using a different pattern of dissection, individual V did show statistically significant variation between ATP levels in the two cell types. To reveal possible gradients within the prespore region

sections were assayed from the interior axis and the peripheral areas of four pseudoplasmodia. Three of these four individuals showed no significant differences in ATP levels between cells from the middle and outer areas of the prespore region.

ATP levels at the culmination stage of development (20h) are shown in Figure 7. Prespore sections contained 7.8 ± 0.8 mmol/kg, while prestalk values ranged from 10.3 ± 0.4 mmol/kg in cells directly adjacent to the prespore mass to 10.8 ± 0.2 mmol/kg in cells at the tip of the prestalk region. Stalk sections revealed a gradient of ATP from 11.8 mmol/kg at the apex of the stalk to approximately 4 mmol/kg in sections near the base.

Mature sorocarps after 24h of development (Figure 8) showed no loss of ATP from spore cells. These differentiating spore cells still had 7.9 ± 0.6 mmol/kg, however, in stalk cells the gradient of ATP declined from 8.59 mmol/kg at the apex of the stalk to 3.52 mmol/kg at the base. In 36h sorocarps ATP had decreased to less than 1 mmol/kg in both cell types (Figure 9). The ATP values observed in 36h sorocarps were not altered by assaying spores which had been ruptured by French pressing (Section 2.4.1). Thus, the weakening of spore coats due to lyophilization was sufficient to allow assay of ATP content.

Cell-specific changes in ATP content during development are summarized in Figure 10. From aggregation to the pseudoplasmodium stage ATP declined from 11.5 ± 2.1 mmol/kg to 10.1 ± 2.5 mmol/kg and 7.16 ± 2.04 mmol/kg in prestalk and prespore cells, respectively.

During the subsequent 6h of development ATP remained at approximately these levels in prestalk and prespore cells. However, the ATP level in stalk cells which had entered the sheath was one third less than the level in prestalk cells at 22h into development. From 22h to 24h ATP levels declined sharply in differentiating stalk cells. Prestalk cells which contained 9.7 ± 1.0 mmol/kg at 22h dropped to 4.1 ± 2.1 mmol/kg at 24h after entering the stalk sheath. During these same 2h of development spore cells showed only a slight decline in ATP concentrations. By 36h after the beginning of starvation both spore and stalk values had decreased to less than 1.0 mmol/kg.

3.2 Trehalose and Glucose

Control experiments were conducted to insure that only trehalose and/or glucose were measured by the procedures described in Section 2.4.2. Prestalk and stalk sections from culmination stage were assayed to detect endogenous trehalase activity. The tissue was assayed as for glucose but trehalose was added to alternate wells. If endogenous trehalase survived the assay procedures it could degrade trehalose and result in higher glucose levels. However, no trehalase activity was detected; therefore the glucose values measured by the assay were not inflated due to degradation of trehalose by endogenous trehalase. Another control experiment determined the level of endogenous G6P detected by the assay. In prestalk sections G6P was insignificant, comprising less than 1% of measured glucose values. In all assays both glucose and trehalose standards were included.

Trehalose and glucose levels were determined in alternate sections of individuals from the pseudoplasmodium, culmination, and 24h sorocarp stages of development. At the pseudoplasmodium stage (16h) both trehalose and glucose averaged 16 mmol/kg in the anterior prestalk region and 4 mmol/kg throughout the posterior prespore region (Figure 11). During the 6h between slug stage and culmination stage, prespore levels of trehalose and glucose remained constant, however, prestalk cells showed pronounced changes in the levels of both substrates (Figure 12). In the prestalk area at culmination, trehalose decreased from 123 mmol/kg to 56 mmol/kg as cells migrated up toward the position for stalk formation. There was a concomitant increase of glucose from 23 mmol/kg to 47 mmol/kg over this path of cell migration. Thus, at this stage, a twentyfold increase in trehalose concentrations was observed between prespore cells and adjacent prestalk cells. Stalk sections of culminating individuals revealed a decreasing gradient of both trehalose and glucose from approximately 70 mmol/kg at the apex of the stalk to less than 5 mmol/kg near the base. In 24h sorocarps (Figure 13), spore sections contained high levels of trehalose (120 mmol/kg) but no significant amounts of glucose. Over a period of 2h (Figures 12 and 13) trehalose increased in prespore cells from 5 mmol/kg to 120 mmol/kg; glucose did not accumulate at all during this time. Both substrates decreased sharply from 35 mmol/kg at the apex of the stalk to less than 1 mmol/kg in the upper portion of the stalk, an area occupied by approximately 400 cells.

3.3 Ammonium Ion

Sections of individuals from five stages of development were assayed for ammonium ion (NH_4^+). Because the reaction mixture alone made a significant contribution to the fluorescence of tissue samples, the sections of tissue assayed for NH_4^+ were tenfold larger than those used in assays of other substrates. In most cases the entire stalk or a comparable section of spore tissue was required for a significant increase over the blank fluorescence.

Several control experiments were conducted for this assay in an effort to discover the source of the high blanks and to determine that only NH_4^+ was being measured. Heating the wells (at 90°C for 10 min) immediately after adding the initial reaction mixture demonstrated that the assay required enzymatic activity; under these conditions no NH_4^+ was detected. The assay was also shown to be dependent on the addition of αKG to the reaction mixture. In searching for the source of the high blank, the ingredients of the first reaction mixture were added one at a time to a series of wells. Most of the blank fluorescence was contributed by the addition of NADPH and, to a lesser extent, GDH.

The data obtained from the assay of NH_4^+ in tissue sections are summarized in Table 3 and Figure 14. At the pseudoplasmodium stage of development the concentration of NH_4^+ in prestalk and prespore sections was 127 ± 28 mmol/kg and 145 ± 37 mmol/kg, respectively. By culmination (20h) these values had dropped to 1.20 ± 2.68 mmol/kg in prespore sections and 49.0 ± 13.4 mmol/kg in prestalk sections.

Cells which had migrated into the stalk showed an NH_4^+ level sixfold higher (313 ± 84 mmol/kg) than prestalk cells which had not yet undergone that phase of differentiation. Stalks of early sorocarps (23.5h) accumulated 625 ± 89 mmol/kg, thus showing a pronounced increase over stalks from culmination-stage individuals. This NH_4^+ level continued to increase to 864 ± 308 mmol/kg in 25h sorocarps and remained at approximately that concentration in 36h fruiting bodies. Whereas the NH_4^+ content increased in stalk tissue between 20h and 23.5h of development, spore tissue did not accumulate high levels until 25h. By 36h of development spore cells had accumulated approximately as much NH_4^+ as stalk cells of that stage.

4. DISCUSSION

4.1 ATP

During development ATP levels in prestalk cells remained constant until cells migrated into the stalk sheath, at which time the cells within the sheath contained one-third to one-half the ATP found in cells which had not yet entered the stalk (Figures 7 and 10). Conversely, ATP levels in prespore cells were constant throughout development. Only after the process of stalk construction was completed and the spore mass had been lifted to the apex of the stalk (36h), did ATP levels decline in spores. By 36h ATP in both spore and stalk cells had dropped to one-tenth the levels found at aggregation.

Farnsworth and Loomis (1974, 1975) have suggested that the increased thickness of the slime sheath at the posterior end of pseudoplasmodia might cause gradients of diffusible metabolites. The ATP levels detected at the pseudoplasmodium stage offer no evidence for the presence of a gradient of ATP either between prestalk and prespore cells or between outer and middle sections of prespore cells. Since no changes in ATP levels were observed early in differentiation, energy availability is probably not a regulatory factor at that time. Diffusion gradients, created by the differential thickness of the sheath or by diffusion of oxygen to cells within the center of a developing individual, if present, are not reflected by detectable differences in ATP levels.

Although cell-specific changes in ATP levels are observed later in development, it is not clear whether these changes result from positional information or the depletion of endogenous substrates. During stalk cell differentiation there is marked evidence of spatial influence as prestalk cells migrate into the top of the forming stalk and concomitantly lose a substantial portion of their ATP content. As these stalk cells enter the stalk sheath, become vacuolized, and produce rigid cell walls, their ATP pools are depleted by the energy demands of this differentiation. The role of spatial information in spore-cell development is less obvious, although as the spore mass is lifted off of the substratum the increased amount of oxygen which can then reach the cells might impart positional information capable of influencing spore development. The pronounced change in ATP content in spore cells occurs only during the final stages of spore development, which include the formation of the spore coat, disappearance of prespore vacuoles (Hohl and Hamamoto, 1969), and approaching dormancy.

Thus, the metabolic balance between ATP-utilizing reactions and ATP-regenerating reactions may shift toward utilization of ATP and, therefore, depletion of ATP pools late in the development of both spore and stalk cells. Although ATP levels are observed to drop in stalk cells long before any changes are apparent in spore cells, the stalk-specific loss probably results from the depletion of total energy sources rather than a shift in energy charge (i.e., a regulatory function). It is known that glycogen is degraded in stalk cells during

this time (Rutherford, 1976) as is trehalose (Rutherford and Jefferson, 1976). Also, although both cell types eventually show similar losses of ATP, the energy charge in the two cell types may be quite different. Stalk cells which, when fully differentiated, are unviable due to complete degradation of cellular constituents, probably deplete all energy reserves. Spore cells, on the other hand, which become dormant but remain viable, may temporarily shift their energy charge by a depletion of ATP and a corresponding increase of AMP. To verify a shift of energy charge in spore cells, it would be necessary to determine the cell-specific levels of AMP and ADP as well as levels of ATP. However, such a study is beyond the scope of this thesis.

4.2 Trehalose and Glucose

Trehalose increased during development from 4 mmol/kg in prespore cells at the pseudoplasmodium stage to 120 mmol/kg in spores of a 24h sorocarp. Most of this thirtyfold increase occurred over the last two hours of development. Glucose in spore cells remained constant at approximately 5 mmol/kg throughout development. In prestalk cells trehalose increased from about 18 mmol/kg at the slug stage to 120 mmol/kg at culmination (22h). During culmination, as cells migrated up toward the position for stalk formation, trehalose declined to 60 mmol/kg. Once cells were within the developing stalk, trehalose levels declined sharply, falling to less than 1 mmol/kg in stalk sections below the spore mass. Glucose in prestalk cells

increased during culmination as cells migrated into position for stalk formation. This increase occurred over the same time period and path of cell movement which revealed the decrease in trehalose concentrations. Glucose levels also decreased quickly after cells entered the stalk.

The accumulation of trehalose in prestalk cells confirms previous findings by Rutherford and Jefferson (1976). The increase in glucose in cells which show a declining trehalose content implies the activity of the degradative enzyme, trehalase, in these cells. This activity has been shown to be localized in prestalk and stalk cells (Jefferson and Rutherford, 1976a). The localization of trehalase in prestalk cells and the lack of the enzyme in prespore cells is also reinforced by the maintenance of high trehalose and low glucose levels in prespore cells during culmination (Figure 13). The reasons for trehalose degradation in stalk cells are unknown at this time. Stalk cells, as they differentiate and become vacuolized, have no need for a storage carbohydrate as a later source of energy. Trehalose and its degradation product, glucose, may be involved in the construction of the stalk as precursors of the structural components since both trehalose and glucose are rapidly lost in stalk cells. Alternatively, both glucose and trehalose may be utilized by stalk cells as energy sources for the final stages of differentiation.

The inverse relationship between trehalose and glucose levels during the development of prestalk cells coupled with knowledge of trehalase activity, implies that the glucose levels rise as a result of

trehalose degradation. This conclusion is not entirely compatible with those of Gezelius (1966) and Wright and Marshall (1971). My results with spore cells could support a model in which glucose pools turnover during development with glucose being produced from G6P by the action of acid phosphatase. However, concerning stalk cell differentiation, it is clear that glucose accumulates as a result of the degradation of trehalose by trehalase. Thus, perhaps, there are two functioning pathways which may lead to production of glucose during development; one is active in spores, the other in stalk cells.

The results reported herein support prior speculation that trehalose acts as a storage carbohydrate in spores of Dictyostelium. Furthermore, the accumulation of trehalose in spores occurs within less than two hours. It is known that glycogen levels decrease from 147 mmol/kg to 1 mmol/kg within two hours (Rutherford, 1976) and this decrease is reflected by a localization of glycogen phosphorylase activity (Harris, 1975). Also, trehalose increases from 6 mmol/kg to 120 mmol/kg within this same two hours; an increase which can be accounted for by increased trehalose-6-phosphate synthetase activity (Jefferson and Rutherford, 1976b). Therefore, it is possible that degradative products of glycogen (G1P) could be used in the synthesis of trehalose (through the actions of phosphoglucomutase and T6P synthetase). The accumulation and retention of high trehalose levels in spores confirm that trehalose is available as an energy source during germination (Cotter and Raper, 1970). However, since glucose levels are low in spores, glucose used as an energy source during

germination must derive from degradation of trehalose or another saccharide.

4.3 Ammonium Ion

During development NH_4^+ accumulated first in stalk cells and later in spore cells. Whereas stalk cells contained 600 mmol/kg by 23.5h into development, spore cells approached this level only after 36h of development. The influence of spatial information can be seen clearly in prestalk and stalk cells. In prestalk cells after 20h of development, NH_4^+ levels were approximately 50 mmol/kg while cells which had entered the stalk by this time had accumulated 300 mmoles of NH_4^+ /kg dry weight.

The sensitivity of the assay for NH_4^+ was limited by the high blank values, which required the use of relatively large sections of tissue. The sources of the high blank appeared to be NADPH and GDH. The blank fluorescence probably resulted from incomplete destruction of the unreacted NADPH, and subsequent cycling of the residual nucleotide, rather than contamination of the NADPH with NADP^+ . The blank fluorescence contributed by GDH was probably due to NH_4^+ bound to the enzyme, since extended dialysis failed to reduce the blank further. The dialyzed GDH caused less blank fluorescence than Norite-treated GDH and, therefore, the contamination was not NADP^+ .

Because of the reduced sensitivity of the NH_4^+ assay, smaller sections of stalk and spore mass could not be assayed. Thus, the migration of cells could not be followed and the presence of gradients

of NH_4^+ accumulation could not be determined. At the pseudoplasmodium stage, where differential sheath thickness or diffusion through the cell mass could cause cell-specific accumulation of NH_4^+ , no differences were observed between the cell types. However, since such large sections of the prespore and prestalk regions were assayed, micro-gradients within a cell type in a developing individual could exist. A more sensitive assay, i.e., capable of assaying much smaller sections, would be required to detect these micro-gradients. During the later stages of development, it is quite likely that gradients in NH_4^+ levels exist. Based on the gradients observed in ATP, trehalose, and glucose levels as reported in this thesis as well as other studies of cell-specific distributions of biochemical events (Jefferson and Rutherford, 1976 a & b; Harris, 1975; Rutherford and Jefferson, 1976; Rutherford, 1976), I would predict an increasing gradient of NH_4^+ down the stalk as these cells complete differentiation.

Stalk cells require energy from protein degradation (as indicated by NH_4^+ accumulation) earlier in development than spore cells. By 20h into development stalk cells are rapidly degrading their protein stores for energy needed for the synthesis of cellulose for cell walls. Spore cells, which begin to degrade large amounts of protein by 23.5h into the life cycle, require energy for completion of spore coats. These observations agree with those of Gregg et al., 1954, and Wright and Anderson, 1960, as to the approximate time of protein utilization and the greater loss of total nitrogen by stalk cells during that time. However, by 36h into development, well after

the formation of mature sorocarps, spore cells have utilized as much protein as stalk cells. Total protein at the pseudoplasmodium stage is estimated at 0.15 mg protein/mg dry weight (Rutherford, unpublished results); if this is divided by the average molecular weight of an amino acid (100), the results indicate that approximately 1.5 moles of NH_4^+ could be derived from the protein sources in one kilogram dry weight. Comparison of this value with the amount of NH_4^+ accumulated during development, 0.5 mol/kg dry weight, indicates that the NH_4^+ bound within the cells and detected by this assay might account for a significant portion of the protein present at the slug stage and degraded during development. In addition to protein sources, ammonium ion may be released by degradation of RNA during development. Also, ammonia is known to be excreted from developing sorocarps (Gregg et al., 1954). Thus, it appears that the toxic properties of excess nitrogen can be neutralized during differentiation by two mechanisms, excretion of ammonia or retention as ammonium salts within the cells.

4.4 Conclusion

The cell-specific levels of ATP, trehalose, glucose, and ammonium ion presented here indicate some basic differences between spore and stalk cell development. Whereas trehalose is preserved in spores, stalk cells utilize trehalose as well as glucose, ATP, and protein late in the developmental process. By approximately 22h into development stalk cells show a definite relationship between spatial information and the metabolism of all four energy-related

substrates investigated. As these cells move up toward the tip of a culminating organism and down into the forming stalk, ATP, trehalose, and glucose (after first increasing as a result of trehalose degradation) decrease. NH_4^+ accumulates, probably in response to protein degradation. Spore cells reveal little effect of spatial information on their metabolism, although they may be influenced by their position on the height of the stalk. The results described in this thesis, although emphasizing the unique developmental characteristics of the two cell types, still leave unanswered the underlying question of whether these metabolic changes occur in response to spatial information or are preprogrammed by transcriptional and translational events.

These observations help to emphasize the need for more cell-specific information concerning the differentiation of Dictyostelium and other developmental models. The regulatory mechanisms of a developing system can be discovered only by relating the biological and biochemical events which occur in specific groups of cells in specific spatial and temporal settings.

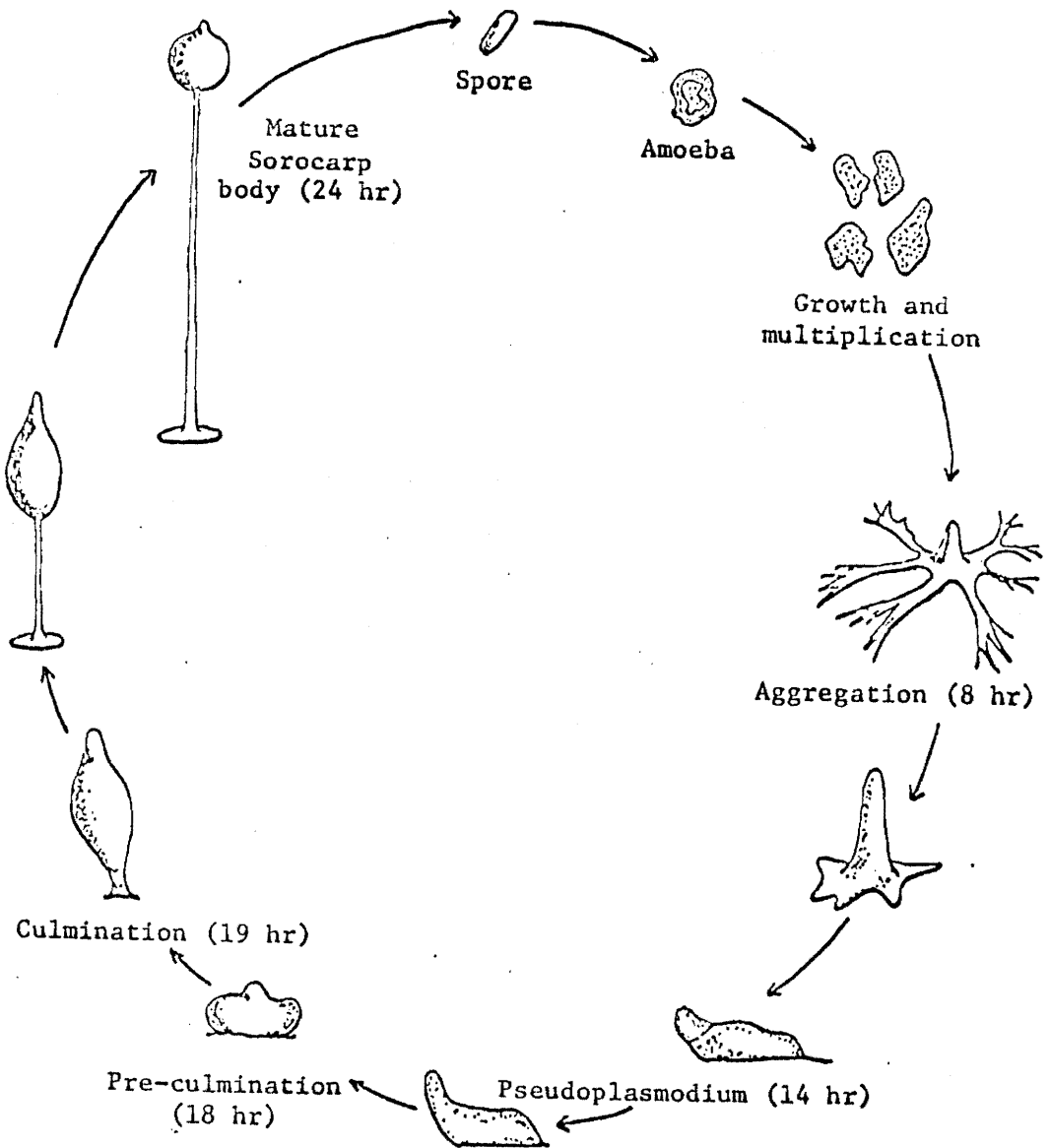
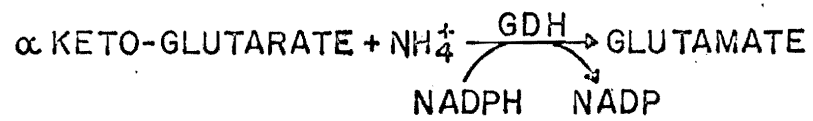
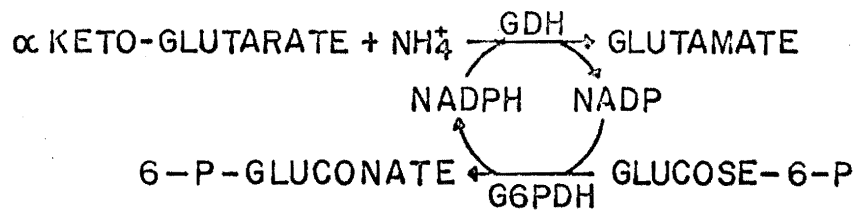


Figure 1. Life cycle of *Dictyostelium discoideum*.

TISSUE REACTION:



CYCLING REACTIONS:



CONVERSION TO PYRIDINE NUCLEOTIDE:

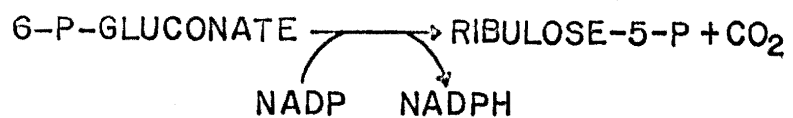
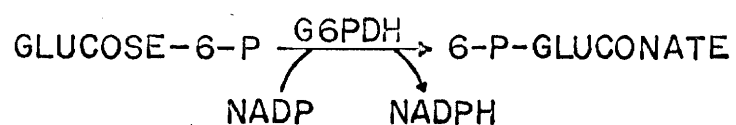
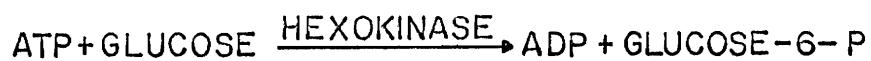
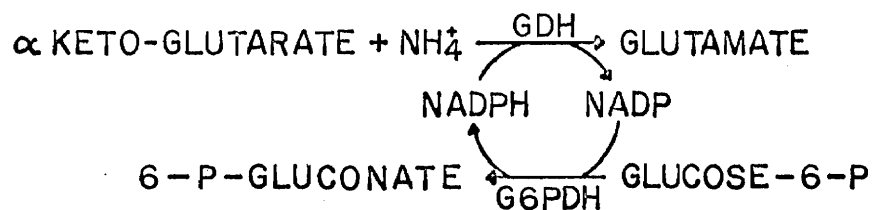


Figure 2. Enzyme Reactions for the Assay of Ammonium Ion.

TISSUE REACTIONS:



CYCLING REACTIONS:



CONVERSION TO PYRIDINE NUCLEOTIDE:

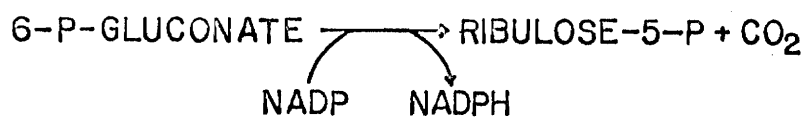


Figure 3. Enzyme Reactions for the Assay of ATP.

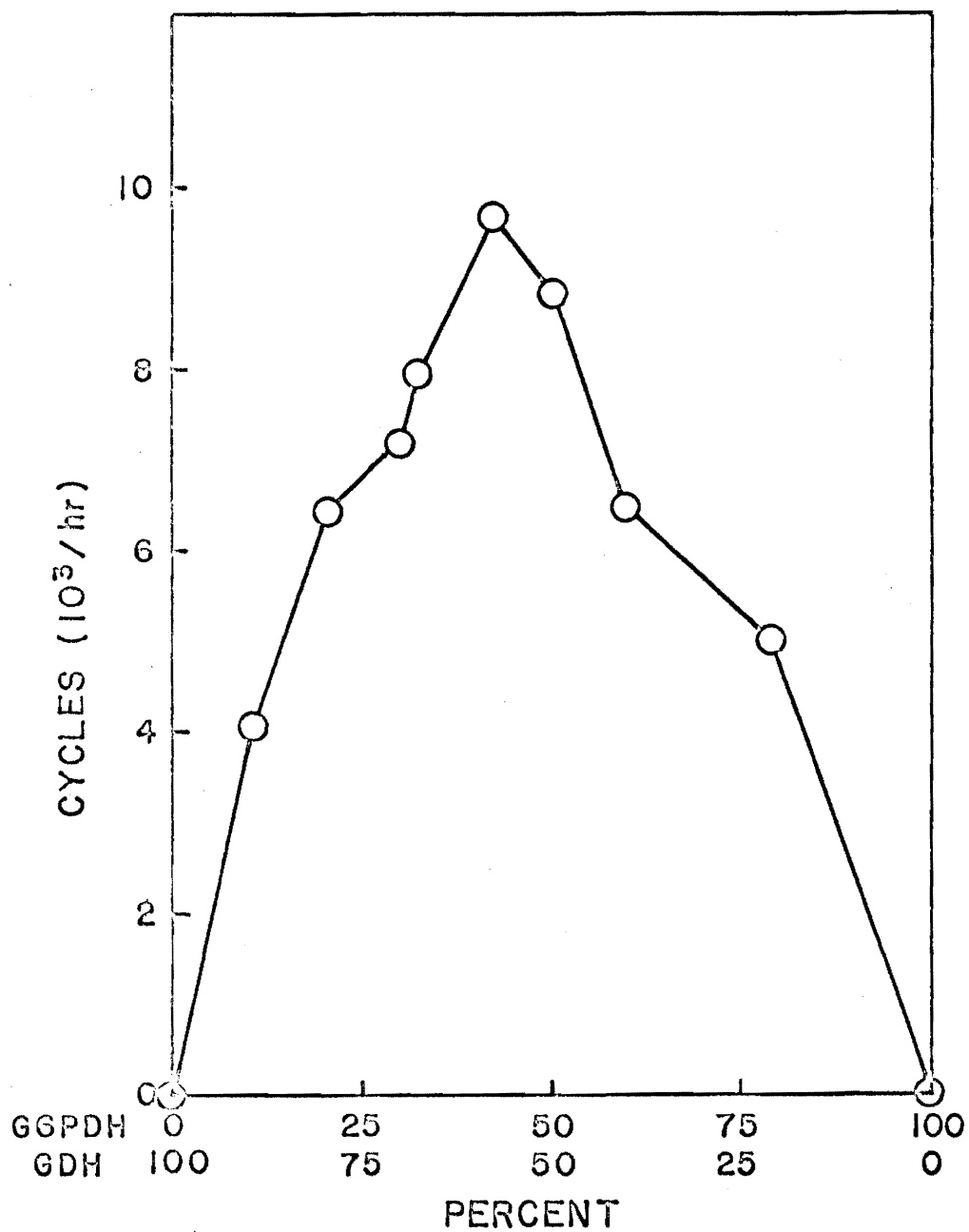
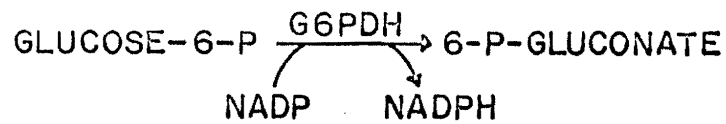


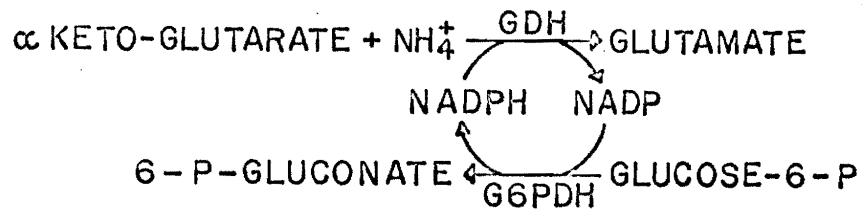
Figure 4. Optimum Ratio of Cycling Enzymes.

Ratios expressed as percentage of G6PDH units to GDH units; total at all ratios was 20 Units.

TISSUE REACTIONS:



CYCLING REACTIONS:



CONVERSION TO PYRIDINE NUCLEOTIDE :

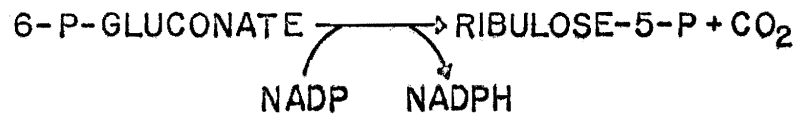


Figure 5. Enzyme Reactions for the Assay of Trehalose and Glucose.

When assaying glucose, trehalase was omitted from the initial reaction mixture.

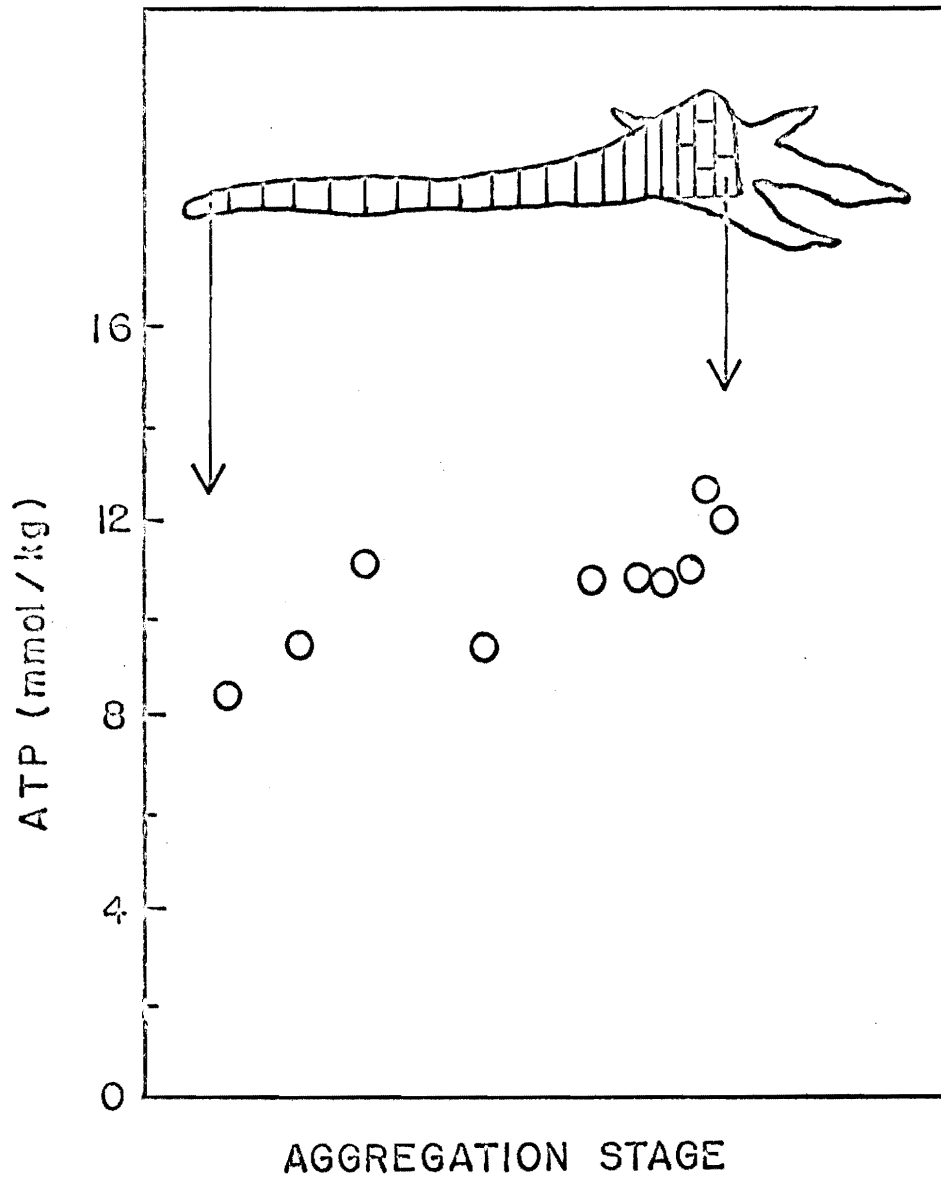


Figure 6. ATP Levels at Aggregation Stage.

Each point represents the average of data from two sections, assayed separately. A total of 3 individuals were dissected, giving 50 sections. The data shown here are from a single representative individual.

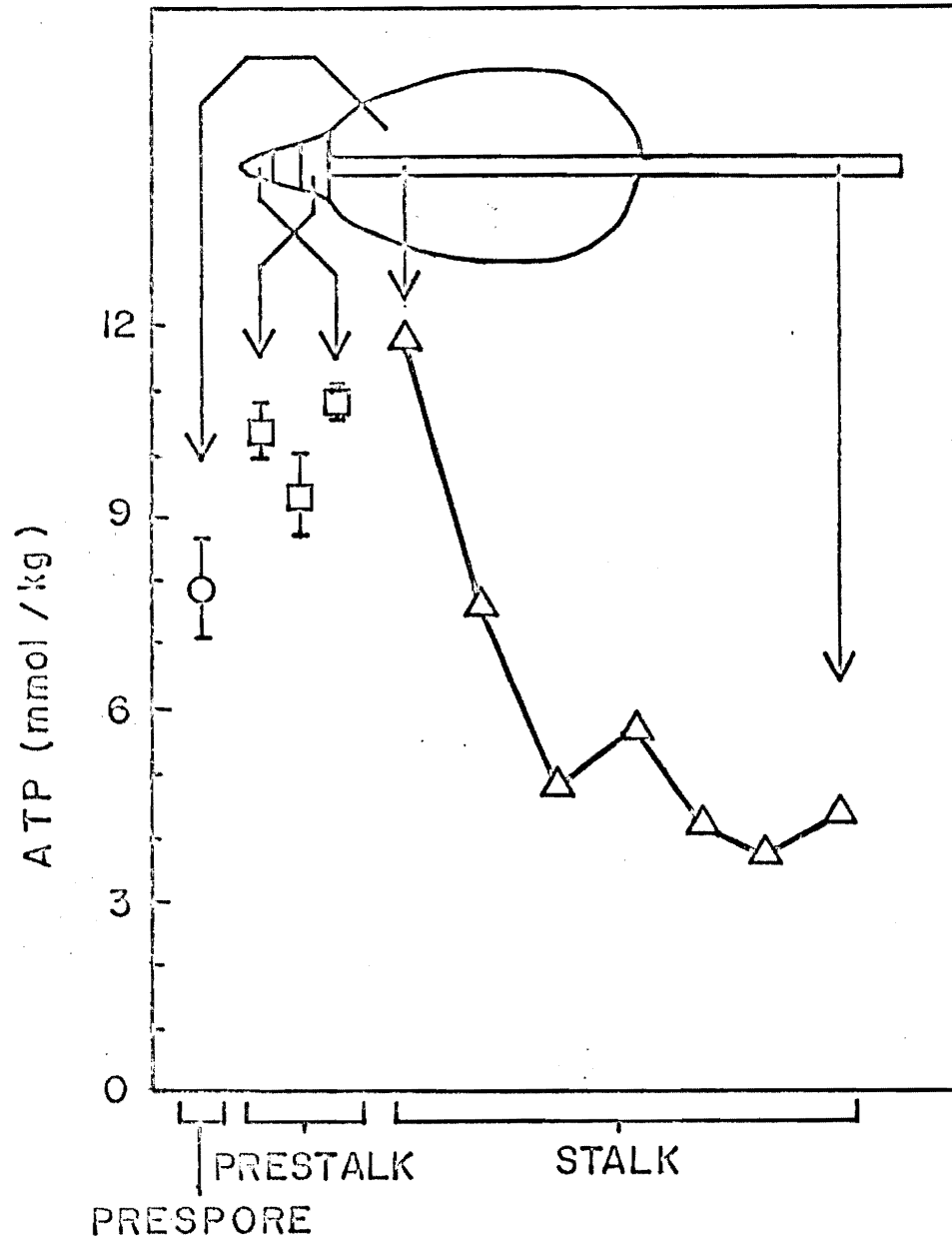


Figure 7. Cell Specific ATP Levels at Culmination Stage.

Circle represents the mean \pm s.d. of 10 prespore sections. Each square represents mean \pm s.d. of 3 prestalk sections. Triangles represent stalk sections. Arrows in the prestalk region indicate that the data is plotted to follow cell migration toward the position for stalk formation. A total of 5 individuals were assayed at this stage; 34 prespore sections, 36 prestalk sections, 27 stalk sections.

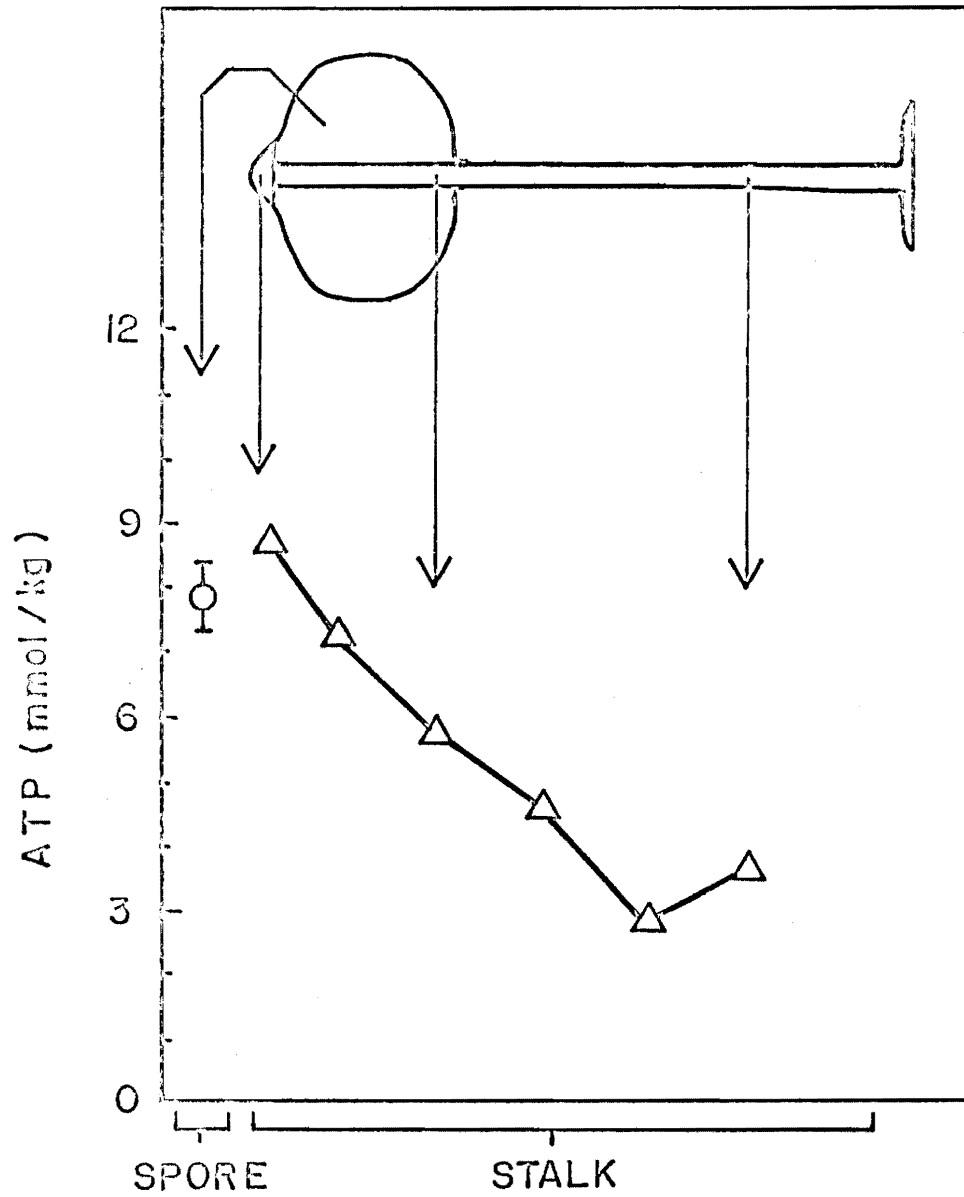


Figure 8. Cell Specific ATP Levels in a 24h Sorocarp.

○ represents the mean \pm s.d. of 10 spore sections. \triangle represents stalk sections. A total of 7 individuals were assayed at this stage; 74 spore sections, 47 stalk sections.

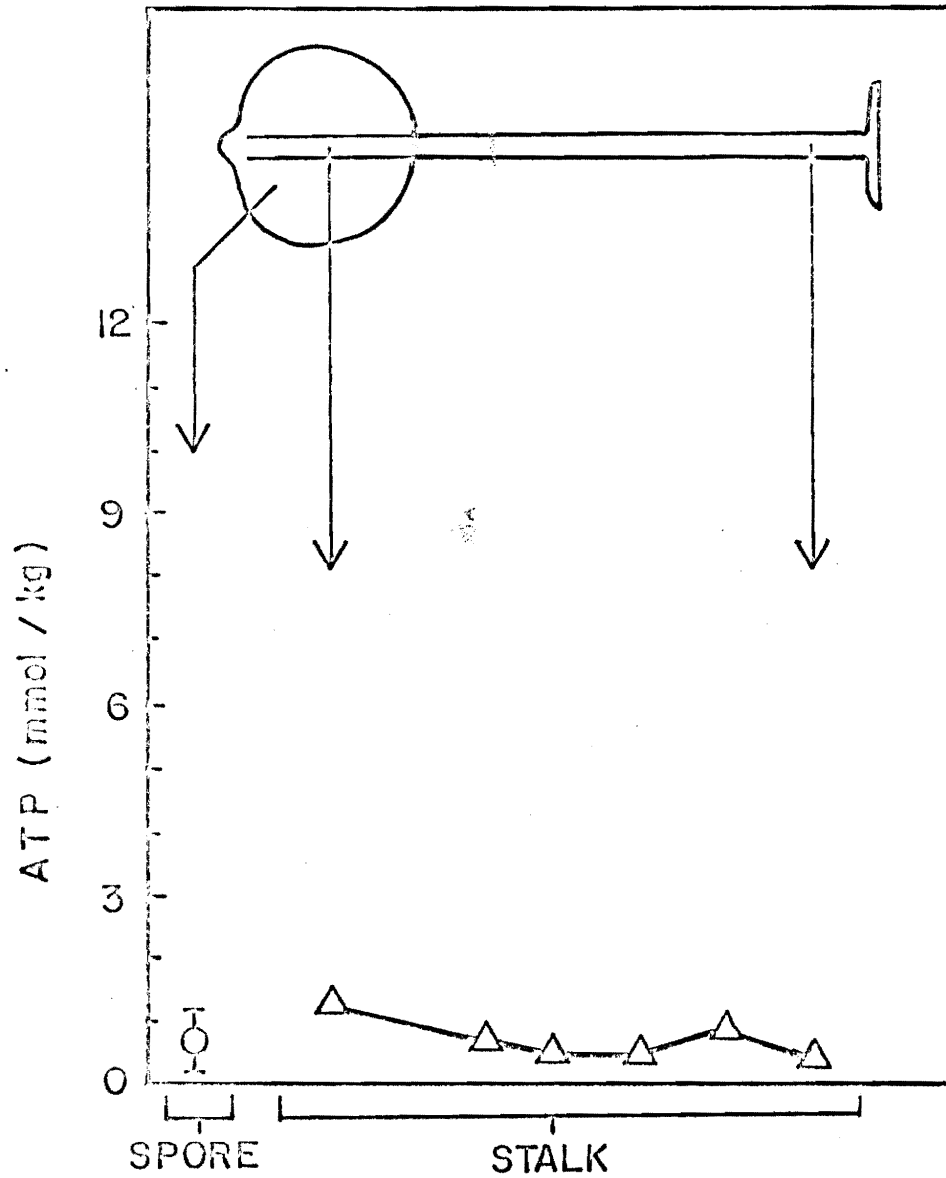


Figure 9. Cell Specific ATP Levels in a 36h Sorocarp.

○ represents mean \pm s.d. of 12 spore sections; \triangle represents stalk sections. A total of 3 individuals were assayed at this stage; 40 spore sections, 22 stalk sections.

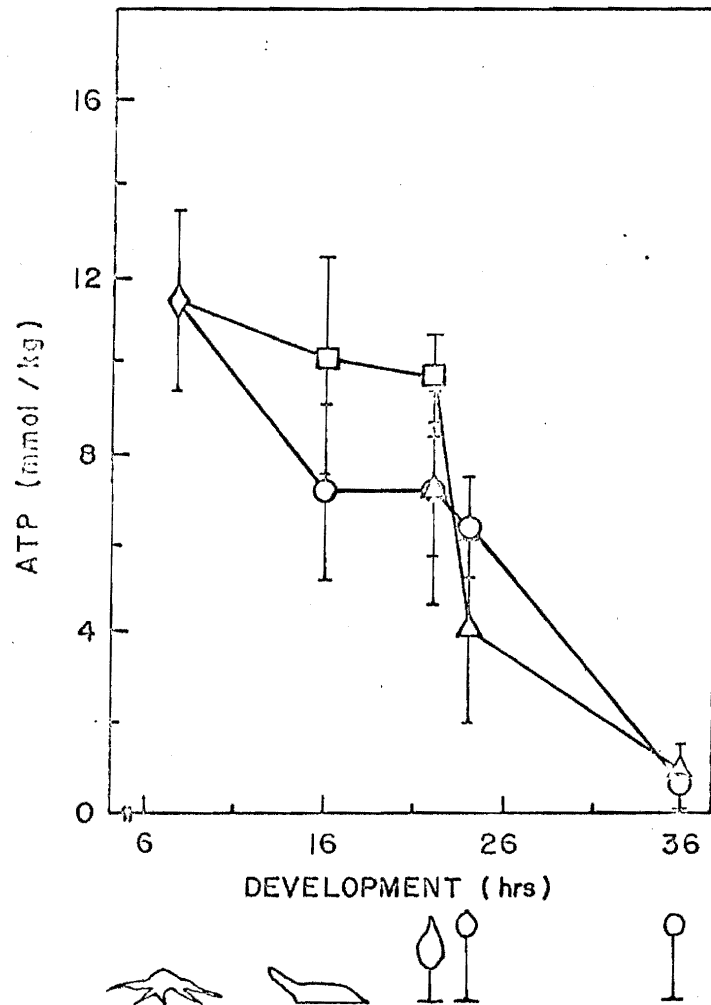


Figure 10. ATP in the Two Cell Types During Development.

aggregation stage, $m \pm$ s.d. of 50 sections (3 indiv.);
 \square = prestalk at slug stage $m \pm$ s.d. of 27 sections (3 indiv.), at 22h $m \pm$ s.d. of 36 sections (5 indiv.); \circ prespore and spore at slug stage $m \pm$ s.d. of 91 sections (5 indiv.), at 22h $m \pm$ s.d. of 34 sections (5 indiv.), at 24h $m \pm$ s.d. of 74 sections (7 indiv.), at 36h $m \pm$ s.d. of 40 sections (3 indiv.); \triangle stalk at 22h $m \pm$ s.d. of 27 sections (5 indiv.), at 24h $m \pm$ s.d. of 47 sections (7 indiv.), at 36h $m \pm$ s.d. of 22 sections (3 indiv.). Weight range for all sections was 0.02-0.15 μ g dry weight.

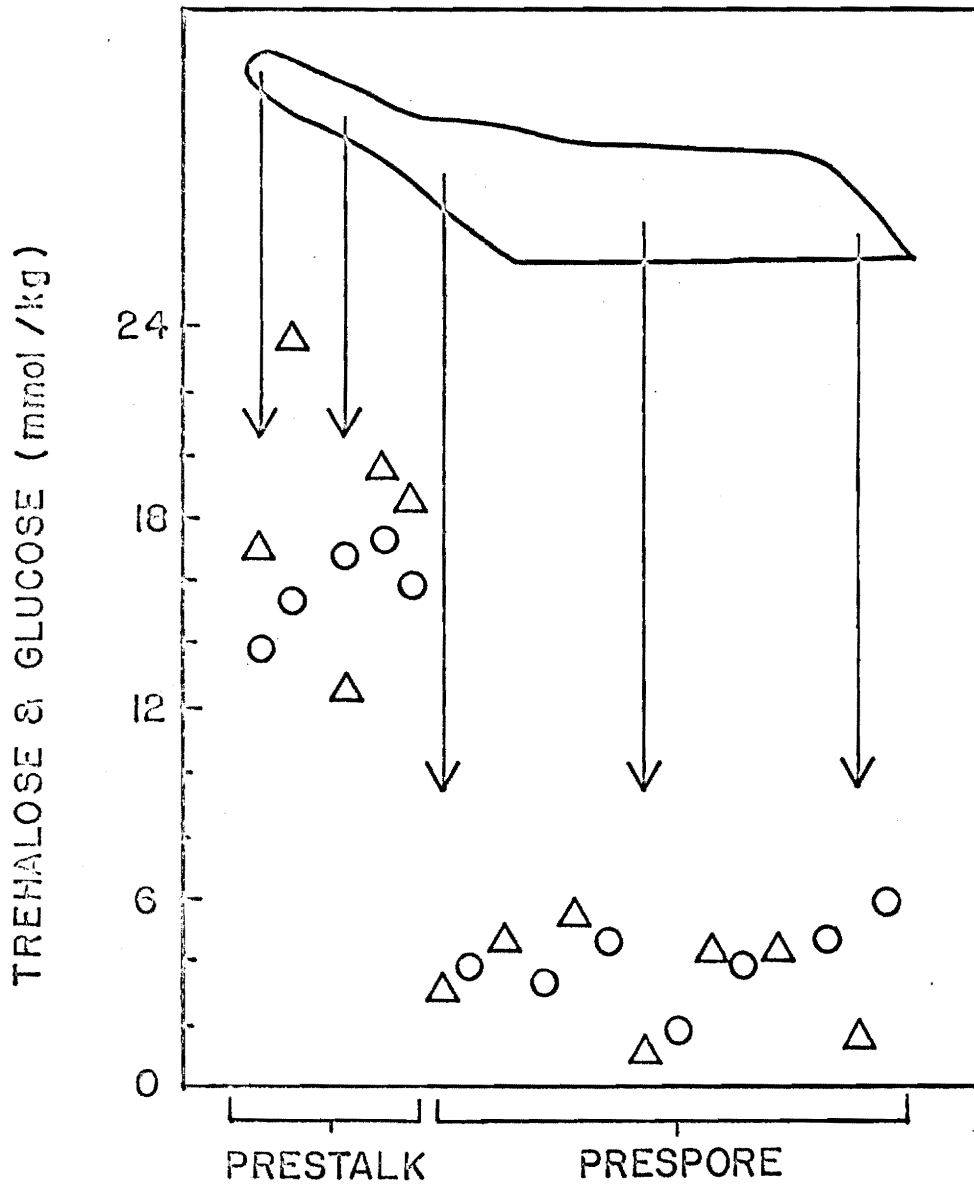


Figure 11. Trehalose and Glucose in Sections of a Pseudoplasmodium.

○ = glucose; △ = trehalose. Sections assayed from an individual at 16h into development. A total of 3 individuals were assayed at this stage; 18 prespore sections and 23 prestalk sections for each substrate. Weight for all sections ranged 0.01-0.07 μ g.

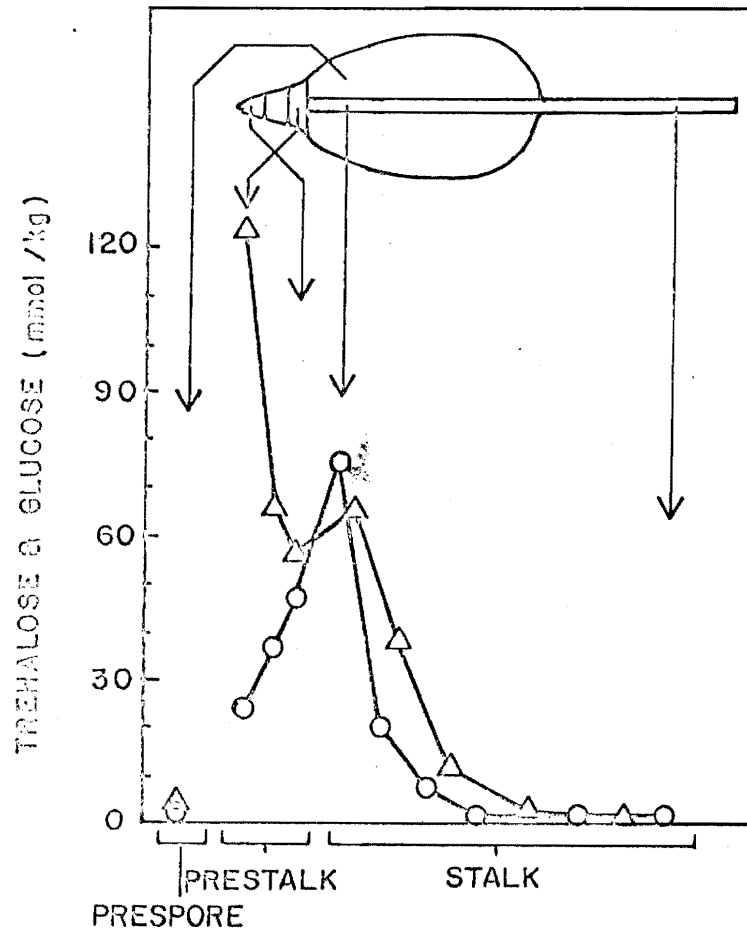


Figure 12. Cell Specific Trehalose and Glucose Levels at Culmination Stage.

○ = glucose; △ = trehalose in an individual at 22h into development. Arrows in the prestalk region indicate that the data is plotted to follow cell migration toward the position for stalk formation. A total of 7 individuals were assayed at this stage; 26 trehalose, 65 glucose prestalk sections; 9 trehalose, 23 glucose prespore and spore sections; 3 trehalose, 16 glucose stalk sections. Weight for all sections ranged 0.01-0.05 μ g.

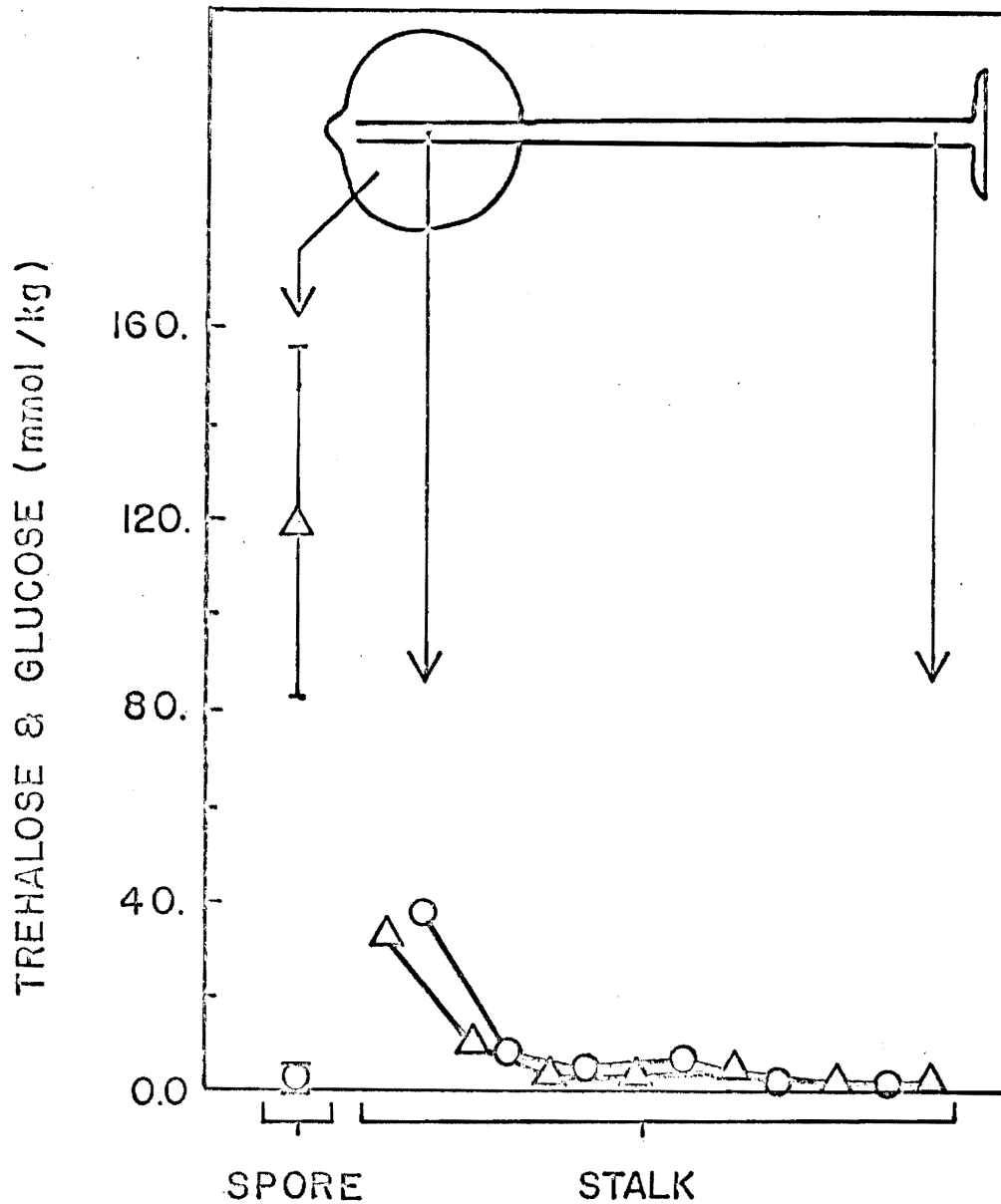


Figure 13. Cell Specific Trehalose and Glucose Levels in a 24h Sorocarp.

○ = glucose, △ = trehalose.

A total of 2 individuals were assayed at this stage; 3 trehalose, 2 glucose sections of prestalk, 10 sections of spore for each substrate, 14 trehalose, 16 glucose sections of stalk. Weight ranged 0.01-0.04 μ g.

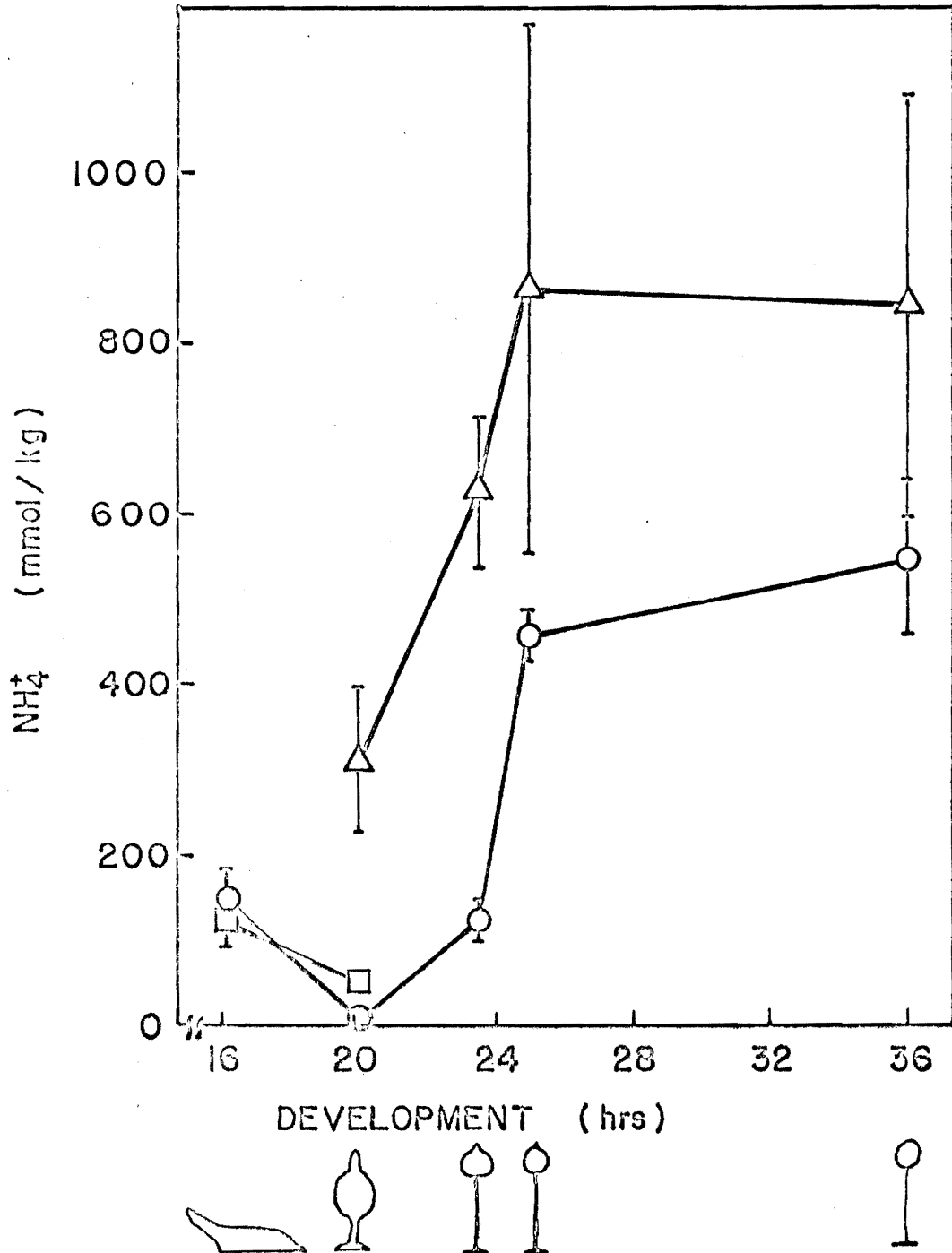


Figure 14. Ammonium Ion Accumulation in the Two Cell Types During Development.

□ = prestalk, ○ = prespore and spore, △ = stalk sections.

Table 1
Detection of Adenosine Nucleotides by the Assay for ATP

Nucleotide (0.25 pmol)	Fluorescence ^c
ATP	8.17 \pm 0.16
ATP + ADP ^a	8.29 \pm 0.20
ATP + ADP + AMP ^b	8.08 \pm 0.12

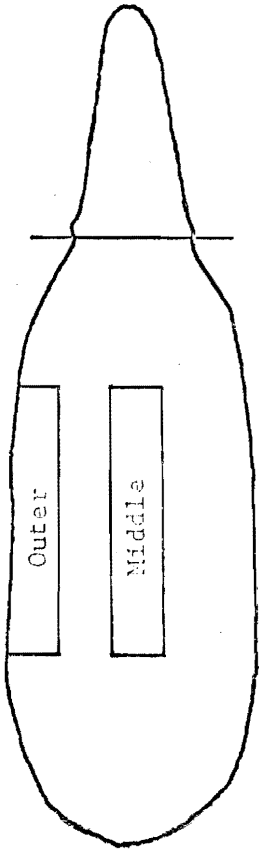
^aEach of four wells contained 0.25 pmol ATP plus 0.25 pmol ADP.

^bEach of four wells contained 0.25 pmol ATP plus 0.25 pmol ADP plus 0.25 pmol AMP.

^cThese values are the mean \pm s.d. of 4 replications.

Table 2

ATP Levels in Sections of Pseudoplasmodia

Cell Types	Individuals Assayed					
	I ^a	II ^a	III ^b	IV ^b	V ^c	
	<u>Prestalk</u>		11.2	7.33	13.2	
			11.4	9.56	13.1	
			8.02	7.07	11.9	
			11.0	7.50	14.5	
			8.30	9.09	13.9	
			5.42	8.01	10.9	
			7.97		11.8	
			11.0		13.0	
			—	—	9.7	
					10.3	
	<u>Prespore</u>					
	Middle	8.63	8.70	3.10	3.03	8.41
		7.80	7.58	6.65	5.00	7.39
		6.50	7.54	3.95	2.35	9.65
		7.89	7.40	4.77	3.24	8.30
		7.89	9.76	8.42	2.78	7.59
		8.84	9.70	8.33	3.64	9.39
		8.83	9.23	6.68		8.84
			5.71	5.67		9.17
				3.39		7.84
		—	—	—	—	7.09
	Outer	3.93	7.56	8.67	6.99	8.33
		3.35	5.43	5.64	8.03	8.96
		8.87	6.40	7.22	7.32	9.28
		9.33	8.66	5.26	5.03	
		4.60	8.91	8.30	4.96	
		7.34	9.28	7.57	7.50	
		8.05	7.76	7.66	6.09	
			8.71	7.44	6.32	
					6.02	

^aMiddle prespore sections were localized linearly from anterior to posterior end; outer prespore sections were dissected randomly from the area indicated in the figure.

^bPrestalk sections were localized linearly from the tip backwards; prespore sections were dissected randomly from the areas indicated in the figure.

^cSections localized from tip backwards over entire length of slug.

Table 3
 Cell Specific Ammonium Ion Levels During Development
 (mmol/kg dry weight)

Stage	Hr of Dev.	# Indiv. Assayed	# Sections Assayed ^{ab}	Cell Type		
				Prestalk	Stalk	Prespore ^c & Spore
Pseudoplasmodium	16	8	8	127 \pm 28	-----	145 \pm 37
Culmination	20	6	6	49 \pm 13.4	313 \pm 84	1.20 \pm 2.68
Early Sorocarp	23.5	9	9	-----	625 \pm 89	123 \pm 23
Sorocarp	25	3	6	-----	864 \pm 308	453 \pm 27
Sorocarp	36	4	4	-----	842 \pm 247	549 \pm 89

^aWeight range for all sections was 0.1-0.9 μ g dry weight.

^bNumber of sections of each cell type.

^cThese are prespore data for the pseudoplasmodium and culmination stages and spore data for all later stages.

REFERENCES

- Atkinson, D. E. 1966. Regulation of Enzyme Activity. *Ann. Rev. Biochem.* 35:85-127.
- Atkinson, D. E. 1968. The Energy Charge of the Adenylate Pool as a Regulatory Parameter. Interaction with Feedback Modifiers. *Biochem.* 7:4030-4034.
- Babloyantz, A. and Hiernaux, J. 1974. Models for Positional Information and Positional Differentiation. *PNAS* 71:1530-1533.
- Bonner, J. T. 1971. Aggregation and Differentiation in the Cellular Slime Molds. *Ann. Rev. Microbiol.* 25:78-92.
- Bonner, J. T., Barkley, D. S., Hall, E. M., Konijn, T. M., Mason, J. W., O'Keefe, G. III, and Wolfe, P. B. 1969. Acrasin, Acrasinase, and the Sensitivity to Acrasin in Dictyostelium discoideum. *Dev. Biol.* 20:72-87.
- Chulavatnatol, M. and Atkinson, D. E. 1973. Kinetic Competition in Vitro between Phosphoenolpyruvate Synthetase and the Pyruvate Dehydrogenase Complex from Escherichia coli. *J. Biol. Chem.* 248:2716-2721.
- Cotter, D. A. and Raper, K. B. 1970. Spore Germination in Dictyostelium discoideum: Trehalase and the Requirement for Protein Synthesis. *Dev. Biol.* 22:112-128.
- Eckert, K., Otto, M., Grosse, R., Heinrich, R., Jacobasch, G. 1975. On the Influence of F-6-P, NH_4^+ , ATP and AMP on the Conformation of the Phosphofructokinase from Rabbit Muscle. *Acta Biol. Med. Germ.* 34:11-17.
- Farnsworth, P. 1973. Morphogenesis in the Cellular Slime Mold Dictyostelium discoideum; the formation and regulation of aggregate tips and the specification of developmental axes. *J. Embryol. exp. Morph.* 29:253-266.
- Farnsworth, P. A. and Loomis, W. F. 1974. A Barrier to Diffusion in Pseudoplasmodia of Dictyostelium discoideum. *Dev. Biol.* 41:77-83.
- Farnsworth, P. A. and Loomis, W. F. 1975. A Gradient in the Thickness of the Surface Sheath in Pseudoplasmodia of Dictyostelium discoideum. *Dev. Biol.* 46:349-357.

- Gadkari, D. and Stolp, H. 1975. Energy Metabolism of Bdellovibrio bacteriovirus. I. Energy Production, ATP Pool, Energy Charge. Arch. Microbiol. 102:179-185.
- Gezelius, K. 1966. Acid Phosphatase in Dictyostelium discoideum. Physiol. Plant. 19:946-959.
- Goodwin, B. C. and Cohen, M. H. 1969. A Phase-shift Model for the Spatial and Temporal Organization of Developing Systems. J. Theor. Biol. 25:49-107.
- Gregg, J. H. 1966. Organization and Synthesis in the Cellular Slime Mold. in The Fungi. Vol II Edited by G. C. Ainsworth and A. S. Sussman. Academic Press, New York.
- Gregg, J. H. and Badman, W. S. 1970. Morphogenesis and Ultrastructure in Dictyostelium. Dev. Biol. 22:96-111.
- Gregg, J. H., Hackney, A. L., and Krivanek, J. O. 1954. Nitrogen Metabolism of the Slime Mold Dictyostelium discoideum, during Growth and Morphogenesis. Biol. Bull. 107:226-235.
- Gustafson, G. L. and Wright, B. E. 1972. Analysis of Approaches used in studying Differentiation of the Cellular Slime Mold. CRC Critical Rev. in Microbiol. 1:453-478.
- Harris, J. F. 1975. Cell-Specific Activities of Glycogen Synthetase and Glycogen Phosphorylase during Development of Dictyostelium discoideum. PhD dissertation. Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
- Hato, M., Ueda, T., Kurihara, K., and Kobatake, Y. 1976. Change in Zeta Potential and Membrane Potential of Slime Mold Physarum polycephalum in response to chemical stimuli. Biochem. Biophys. Acta 426:73-80.
- Hohl, H. R. and Hamamoto, S. T. 1969. Ultrastructure of Spore differentiation in Dictyostelium: The Prespore Vacuole. J. Ultrastruc. Res. 26:442-453.
- Jefferson, B. L. and Rutherford, C. L. 1976a. A Stalk Specific Localization of Trehalase Activity in Dictyostelium discoideum. Exptl. Cell. Res. in print.
- Jefferson, B. L. and Rutherford, C. L. 1976b. Cell Specific Activity of Trehalose-6-phosphate Synthetase during Differentiation of Dictyostelium discoideum. Cell Differentiation in print.

- Krivanek, J. O. and Krivanek, R. C. 1958. The Histochemical Localization of Certain Biochemical Intermediates and Enzymes in the Developing slime Mold Dictyostelium discoideum. J. Exptl. Zool. 137:68-115.
- Liddel, G. U. and Wright, B. E. 1961. The Effect of Glucose on Respiration of the Differentiating Slime Mold. Dev. Biol. 3:265-276.
- Loomis, W. F., Jr. 1972. Role of the Surface Sheath in the Control of Morphogenesis in Dictyostelium discoideum. Nature New Biol. 240:6-9.
- Loomis, W. F. 1975. Dictyostelium discoideum. A Developmental System. Academic Press, New York.
- Lowry, O. H. and Passonneau, J. V. 1972. A Flexible System of Enzymatic Analysis. Academic Press, New York.
- Otto, M., Heinrich, R., Kuhn, B., and Jacobasch, G. 1974. A Mathematical Model for the Influence of fructose-6-phosphate, ATP, Potassium, Ammonium, and Magnesium on the Phosphofructokinase from Rat Erythrocytes. Eur. J. Biochem. 49:169-178.
- Raper, K. B. 1940. Pseudoplasmodium Formation and Organization in Dictyostelium discoideum. J. Elisha Mitchell Scien. Soc. 56:241-282.
- Raper, K. B. and Fennell, D. I. 1952. Stalk Formation in Dictyostelium. Bull. Torrey Bot. Club 79:25-51.
- Rubin, J. and Robertson, A. 1975. The Tip of the Dictyostelium discoideum pseudoplasmodium as an organizer. J. Embryol. exp. Morph. 33:227-241.
- Rutherford, C. L. 1976. Cell Specific Events occurring during Development. J. Embryol. exp. Morph. 35:335-343.
- Rutherford, C. L. and Jefferson, B. L. 1976. Trehalose Accumulation in Stalk and Spore Cells of Dictyostelium discoideum. Dev. Biol. in print.
- Tabor, C. W. 1970. Determination of NH_3 with the Use of Glutamic Dehydrogenase. in Methods in Enzymology. Vol XVII A. pg 955. ed. H. Tabor and C. W. Tabor. Academic Press, New York.

- Tager, J. M., Akerboom, T. P. M., Hoek, J. B., Jeijer, A. J., Vaartjes, W., Ernster, L., Williamson, J. R. 1975. Ammonia and Energy Metabolism in Isolated Mitochondria and intact liver cells. in Normal and Pathological Development of Energy Metabolism. Academic Press, London.
- Watts, D. J., Treffry, T. E. 1975. Incorporation of N-acetylglucosamine into the Slime Sheath of the Cellular Slime Mould Dictyostelium discoideum. FEBS Letters 52:262-264.
- Wolpert, L. 1969. Positional Information and the Spatial Pattern of Cellular Differentiation. J. Theor. Biol. 25:1-47.
- Wright, B. E. and Anderson, M. L. 1960. Protein and Amino Acid Turnover During Differentiation in the Slime Mold. I. Utilization of Endogenous amino acids and proteins. Biochim. Biophys. Acta 43:62-66.
- Wright, B. E. and Marshall, R. 1971. Trehalose Synthesis during Differentiation in Dictyostelium discoideum. I. Analysis and predictions by computer simulation. J. Biol. Chem. 246:5335-5339.

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ATP, TREHALOSE, GLUCOSE, AND AMMONIUM ION
LEVELS IN THE TWO CELL TYPES OF
DICTYOSTELIUM DISCOIDEUM

by

Jeanne Burrowbridge Wilson

(ABSTRACT)

Ultra-microfluorometric techniques were adapted to follow several compounds related to energy metabolism through the developmental cycle of Dictyostelium discoideum. Each compound (ATP, trehalose, glucose, and ammonium ion) was found to be present in stalk and/or spore cells.

The accumulation of NH_4^+ was interpreted as an indication of protein degradation, a source of energy in this organism. During the early stages of differentiation NH_4^+ was localized only in stalk cells. However, it accumulated in spore cells during culmination such that levels were comparable in the two cell types by the end of development. Trehalose, an energy source for germinating spores, was found in both cell types but was preferentially degraded in stalk cells late in development. Glucose, the degradation product of trehalose, was localized in stalk cells and varied inversely with trehalose in prestalk cells. ATP was not localized in a specific cell type during development. However, ATP declined in stalk cells at an earlier

stage of development. These findings emphasize the need for knowledge of cell-specific events involved in the differentiation of this and other organisms.