METABOLIC PATTERNS AND NUTRITIONAL EFFECTS ON DIFFERENTIATION (SCLEROTIZATION) IN THE MYXOMYCETE, PHYSARUM FLAVICOMUM

A Thesis

Presented to

The Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Glenna Sue Maxey

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METABOLIC PATTERNS AND NUTRITIONAL EFFECTS ON DIFFERENTIATION (SCLEROTIZATION) IN THE MYXOMYCETE, *PHYSARUM FLAVICOMUM*

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ABSTRACT

The subject of this research endeavor is the determination of the nutritional effects and biochemical events associated with the differentiation (sclerotization) of the Myxomycete Physarum flavicomum variety 1. During this differentiation process, the formerly growing, naked, acellular plasmodium converts into numerous dormant cells surrounded by cell walls. It has been generally assumed that starvation conditions are necessary for the induction of differentiation. This work, however, establishes that, not complete starvation, but a condition of nutrient imbalance triggers the differentiation process. That is, the unavailability of an adequate spectrum of amino acids in the medium initiates the myriad of metabolic and morphological alterations characteristic of the sclerotizing plasmodium. In the absence of extracellular amino acids, cellular amino acids and cellular protein are catabolized, thereby diminishing these cell fractions, and sclerotization proceeds. However, in the presence of extracellular amino acids, sclerotization is delayed, and the amino acid content of the soluble pool and protein content of the incubating cells are comparable to those of the respective fractions of plasmodia under normal growth conditions.

A number of nutritional factors are studied for their effect in influencing the sclerotization process. Sclerotization readily occurs in the presence of an adequate supply of glucose. The degradation of cellular protein also occurs even when glucose is available in sclerotization medium. During sclerotization in the presence of dimethyl sulfoxide,

protein degradation again occurs quite readily with a rapid decrease in the amino acid content of the cell soluble pool. On the other hand, certain concentrations of ammonium ions, when present in sclerotization medium, affects a decreased rate of protein degradation and sclerotization. The amino acid content of the cell soluble pool, however, does decrease.

This research also establishes that active transport systems for amino acids remain functional in P. flavicomum during sclerotization. When certain amino acids are included in sclerotization media, they are rapidly accumulated in the cell pool and protein fractions. Radioactive assay of such accumulation shows that an initial burst of protein synthesis is necessary for the formation of sclerotia and occurs during the initial stage of this differentiation. The majority of this protein is degraded shortly after its synthesis. The protein synthesized during active growth of cells in the presence of an adequate supply of nutrients has similarly been shown to be steadily degraded in these differentiating cells.

Sclerotization (differentiation), therefore, is characterized by a change in the metabolism of the sclerotizing plasmodium, as cell amino acids and cell proteins are actively catabolized during the formation of the dormant cells. This preferential metabolism of amino acids during sclerotization thus metabolically resembles the differentiation process in the cellular slime molds and certain bacterial and fungal systems.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

INTRODUCTION

The Myxomycetes, or acellular slime molds, have been known and studied for many years for their unique life cycle. Physarum flavicomum variety 1 constitutes one species of these slime molds. The somatic stage of P. flavicomum is a free-living, multinucleate, motile mass of protoplasm, the plasmodium. The plasmodium is the assimilative, most characteristic phase of the organism's life cycle. This stage of P. flavicomum is found to vary in size and morphology, the relatively small cells with few nuclei formed in liquid, shake culture being referred to as microplasmodia. The plasmodium contains a yellow pigment, has no cell wall, and is irregular in shape.

Although many biochemical studies of this slime mold are concerned with the growing plasmodia, differentiation of the plasmodia to form sclerotia is little understood biochemically. The formation of sclerotia, while not a necessary part of the organism's life cycle, is important to the survival of this organism. Sclerotization occurs only after plasmodial growth has ceased (1). When subjected to adverse environmental conditions, specifically starvation, the dormant sclerotia are formed. The sclerotia are hardened bodies containing many small, closely packed spherules within the newly formed cell wall. According to Jump (2), sclerotization is an orderly process in which the following events occur: 1) streaming of the protoplasm ceases; 2) the cell structure gels; 3) the cell wall is formed; and 4) the nuclei are distributed among the spherules. These changes are accompanied by darker pigmentation in the cells.

Biochemical studies involving the sclerotization process in P. polycephalum have recently been reported. McCormick, et al (3) have analyzed the cell wall of the spherules formed during sclerotization. Their studies revealed the presence of a single sugar, galactosamine. The cell walls are also reported to contain 2% protein and a single glycoprotein. The unique presence of the galactosamine in the walls suggests that this slime mold is not related to other groups of organisms (fungi or protozoa) that have been examined (3). Hutterman, et al (4), have also contrasted the activities of seven enzymes in the plasmodial stage with those found in the sclerotia of this acellular slime mold. Most interesting is their report of decreased activity of glucose-6-phosphate dehydrogenase, an enzyme involved in the pentose phosphate pathway, during sclerotization. Meanwhile, glutamate dehydrogenase is observed to increase in activity (4). The latter enzyme plays a primary role in glutamic acid catabolism. Additionally, Sauer, et al, (5), have reported decreases in cell protein and nucleic acid in sclerotizing P. polycephalum cells. Furthermore, sclerotization of this organism has been shown to result in decreased content of glycogen, long chain polysaccharides, reducing sugars, and mucoproteins with an increase in lipids (6, 7). In addition to these discoveries involving sclerotization of P. polycephalum plasmodia, Lynch and Henney (8) have demonstrated a decrease in carbohydrate metabolism in sclerotizing P. flavicomum cells. When compared to growing plasmodia, they also found that the activity of the Embden-Meyerhof-Parnas (EMP) pathway is reduced to a greater extent than that

of the pentose phosphate pathway.

Although these biochemical characteristics have been determined for Physarum during differentiation, there is a paucity of information available concerning the possible metabolic changes occurring during sclerotization. The exact mechanisms of differentiation are complex and quite controversial. Differentiation has been interpreted as being controlled by transcription, translation, and/or general metabolic changes remote from gene expression (5, 9). Nevertheless, it is probably influenced to some extent by the availability of nutrients in the medium which in turn affect the concentration of intermediary metabolites in systems such as Physarum. The amount and pattern of the low molecular weight metabolites of the cells are believed to be a direct reflection of the cells' adaptation to environmental conditions which promote either cell growth or differentiation (10-15). As a consequence, the amino acid pool components of the vegetative phase as well as those of the differentiated phase have been specifically analyzed in a variety of bacterial and fungal systems (10-14). The reported patterns of fluctuation in these pools of low molecular weight metabolites and the increased activity of proteolytic enzymes with differentiation as well as recent investigations of cell transport systems have prompted this study of sclerotization in P. flavicomum. The amino acid pool content as well as cell protein of sclerotizing plasmodial cells, under a variety of conditions affecting the differentiation process, are, thus, carefully scrutinized to more fully understand the mechanism of differentiation in this acellular slime mold.

MATERIALS AND METHODS

Organism and Growth Medium

The Myxomycete, Physarum flavicomum variety 1, is maintained in pure shake culture in a soluble, semi-defined medium developed by Henney and Henney (15). Minimal growth requirements for this species have been described by Henney and Lynch (16).

Prior to cell transfer, 200 ml of the semi-defined medium are sterilized in 500 ml, cotton-plugged, Erlenmeyer flasks, and 0.2 ml (1% (v/v)) of a sterile 0.25% (w/v) hematin (Sigma Chem. Co., St. Louis, Mo.) solution in 1% (w/v) NaOH (J.T. Baker Chem. Co., Phillipsburg, N.J.) is added (15). From a well dispersed culture of cells in maximum growth phase (prior to secretion of slime), 1 1/2 ml of microplasmodia are transferred to each flask of medium. The cultures are grown in a dark room at 25°C for approximately 4 days on a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 170 r.p.m.

Sclerotization Conditions

A dormant stage in the organism's life cycle, sclerotization, is commonly induced by culture in starvation media. A sterile, 1 X basal salts solution is used (8). Growth media is aseptically decanted from presettled cells that are in maximum growth yield. Two hundred ml sterile basal salts are used to wash the cells. The microplasmodia are then resuspended in 200 ml basal salts and placed back on a shaker. Sclerotization is complete in approximately 48 h to 72 h.

Morphological Observations

Several different chemicals, when added to basal salts, alter the rate of sclerotization of P. flavicomum, either to inhibit or accelerate the process. Those cells observed are grown in shake culture in semi-defined medium and then transferred to basal salts including one of the following (final concentrations are indicated):

0.5% (w/v) casein hydrolysate (Table I) (Sheffield Chem. Co., Union, N.J.)

0.5% (w/v) dextrose (Fisher Scientific Co., Fairlawn, N.J.)

1% (v/v) dimethyl sulfoxide (DMSO) (Matheson, Coleman & Bell, Norwood, Ohio)

1 mM aminonicotinamide (Sigma Chem. Co.)

12.4 mM ammonium nitrate (J.T. Baker Chem. Co.)

1 mM diamide (Nutritional Biochemical Corp., Cleveland, Ohio)

0.2 mM Cyclic Adenosine Monophosphate Nucleotide (C-AMP) (Sigma Chem. Co.)

0.25 mM p-phenylazo-phenyl-carbamyl-choline-iodide (PI) (Nutritional Biochemicals Corp.)

Cells transferred to basal salts are used as a basis for comparing the effect of these added compounds on sclerotization. Parameters compared include color intensity, cell wall formation, streaming activity of the cells, their forming a round structure (in contrast to the elongated, irregular shaped microplasmodia), and spherule organization within the microsclerotia. Observations are made at 24 h intervals for 72 h and/or 96 h.

Table 1. Composition of casein hydrolysate included in basal salts medium.[a]

Amino Acid[b]	mg/g	Molecular Wt.	Mole/g	Mole Percent
Lysine	57.40	146.2	.39	18.13
Histidine	6.19	155.2	.03	1.39
Arginine	28.20	174.2	.16	7.44
Aspartic Acid	2.57	133.1	.01	.46
Threonine	11.10	119.1	.09	4.18
Serine	11.80	105.1	.11	5.11
Glutamic Acid	7.92	147.1	.05	2.32
Proline	1.68	115.1	.01	.46
Glycine	2.55	75.1	.03	1.39
Alanine	6.30	89.1	.07	3.25
Cystine	---	---	---	---
Valine	20.30	117.1	.17	7.90
Methionine	15.70	149.2	.10	.46
Isoleucine	10.40	131.2	.07	3.25
Leucine	74.80	131.2	.57	26.51
Tyrosine	14.40	181.2	.07	3.25
Phenylalanine	29.10	165.2	.17	7.90
Tryptophan	11.10	204.2	.05	2.32

a data on casein hydrolysate supplied by Sheffield Chemical Co.
b ammonium concentration is insignificant (.295 mg/g).

Photomicrographs of the sclerotizing plasmodia are taken after 48 h incubation in their respective salts mixture. Panatomic X-135 (Eastman Kodak Co., Rochester, N. Y.) is used.

Initially, cultures were placed on a shaker at 170 r.p.m. Shaker rotation is slowed to 140 r.p.m., however, to prevent rupturing of sclerotia which leads to some of these cells reverting to an active state between 72 and 96 h. The lower rotation speed prevents cell wall breakdown and release of nutrients, thought to induce reversion. After 96 h, microsclerotia are also transferred to semi-defined media to affirm viability.

Preparation of Soluble Amino Acid Pool

The morphological observations, especially those of plasmodia incubated with inorganic and organic nitrogen sources, have subsequently led to an analysis of the amino acid pools of this organism cultured under these conditions. The soluble amino acid pools of the growing microplasmodia and 48 h old, basal salts-induced sclerotia are determined, and used as a basis for comparing those cells incubated in basal salts containing various other compounds. Specifically basal salts containing 0.5% (w/v) dextrose, 1% (v/v) DMSO, 12.4mM ammonium nitrate, or 0.5% (w/v) casein hydrolysate are used as sclerotizing media for plasmodia since they are morphologically observed to affect the differentiation process. Cells sclerotizing in basal salts with casein hydrolysate are also studied for pool fluctuation after 96 h incubation.

Microplasmodia in maximum growth phase are harvested for pool analysis; microsclerotia are harvested after 48 h in their salts medium. Also cells in basal salts with casein hydrolysate are collected at 96 h.

These presettled cells are decanted into 250 ml polypropylene, tared centrifuge bottles (Curtin Chem. Co., Houston, Texas) and centrifuged in a Sorvall model RC2-B refrigerated centrifuge (Ivan Sorval, Inc., Norwalk, Conn.) at 14 500 x g for 5 min at 2°C. The media is poured off. The cell pellet is gently washed with double distilled water and the wash is discarded. The bottle with cells is weighed. The wet weight of the cells collected is determined, and approximately 10 to 15 g used for pool analysis. There are approximately 7 g (wet weight) of cells per 200 ml media. These cells may be frozen or treated immediately for analysis of soluble pool. If frozen, cells are thawed quickly prior to use. All procedures are performed in an ice bath unless otherwise stated. The procedures for preparation is a modification of the method reported by Moore and Stein (17).

Cellular protein is precipitated by suspending cells in 15 x volume of 1% (w/v) picric acid (Matheson, Coleman & Bell). The rigidity of the sclerotial cell wall dictates the method of cell breakage. Plasmodia with fragile cell membranes, are homogenized in picric acid in a 100 ml glass homogenizer to approximately 85% to 90% breakage. With sclerotia, this treatment gives 50% breakage. Microsclerotia, therefore, are suspended in 15 x volume of picric acid in a 2-speed Waring Blender (Model 1042) and then burst by exposure to N_2 at 2000 p.s.i. in a stainless steel Parr 4625 Cell Disruption Bomb (Parr Instrument Co., Moline, Ill.) for 30 min at 4°C. This gives approximately 80% to 90% cell breakage. These suspensions are centrifuged at 14 500 x g for 10 min to precipitate proteins.

To precipitate small polypeptides, a 1 h centrifugation is necessary. At 4°C with a #30 fixed angle rotor, the supernatant from the 14 500 centrifugation is centrifuged at 50 000 X g in a Beckman Model L3-50 Ultracentrifuge (Beckman Instrument, Inc., Fullerton, Cal.). The supernatant is collected and then layered with 2 volumes of cold acetone (Mallinckrodt Chem. Works, St. Louis, Mo.) to precipitate any plasmodial slime. This is not necessary with sclerotizing plasmodia as cellular slime has been secreted. The solution is slowly stirred with a glass rod, winding out the viscous slime (18). To precipitate small particles of slime, the acetone solution is then centrifuged for 10 min at 15 000 X g at 2°C . The supernate, the picric acid-acetone solution, is flash evaporated to one-fourth volume on a rotary evaporator (Buchler Instruments, Fort Lee, N.J.) at 50°C. The boiling flasks containing sample are precleaned by immersion for at least 24 h in an acid cleaning solution (containing 82% concentrated HCl (Matheson, Coleman & Bell) and 18% concentrated nitric acid (Matheson, Coleman & Bell)), rinsed 5 times each with tap and deionized water and then with glass distilled water. The glassware is oven-dried.

An anion exchange resin, AG2X-10 (Biorad Laboratories, Richmond, Cal.), is used to remove the picric acid. Depending on sample size, 16 to 20 g of the resin are washed four times in double distilled water. With the last washing, the resin is allowed to settle and is degassed for 30 min. At least 12 h before use, a Pyrex chromatographic column (Ace Glass, Inc., Vineland, N.J.) is vertically aligned. Approximately 15 ml double

distilled water is pipetted into the column, and slurries of resin are pipetted below the liquid surface so as to prevent trapping air. The outlet is opened after several ml of resin has been added to the column and is closed when all the resin has been added and the water miniscus is a few centimeters above the packing surface. The resin settles by gravity. To prevent any possible residual slime from being carried over to the column, a small amount of glass wool is added to the top of the packed column. To avoid disturbing the resin surface, the sample is applied first with capillary tubing to a few centimeters and then is pipetted, letting it slowly flow down the column sides. The effluent is collected dropwise. The column is washed five times with 3 ml of 0.02 N HCl. Hydrochloric acid is removed from the sample by flash evaporating the wash to dryness. The sample is then added to the same flask and dried. Flasks are washed with pH 2.2 buffer (Table 2) in quantities giving a final concentration of extract equivalent to approximately 1 g (wet weight) cells per ml of buffer for amino acid analysis. The prepared solution of amino acids can be used immediately or stored for several months frozen at -20°C.

Table 2. Sodium citrate buffers used in amino acid analyzer.

Component	Buffer pH[a]				
	2.2±0.03 Sample Dilutor	3.30± 0.01 Column 1	4.25±0.02 Column 1	4.21±0.02 Column 2	5.28±0.02 Column 2
Sodium Concentration	0.20N	0.20N	0.20N	0.38N	0.35N
Citric Acid·H_2O (J.T. Baker Chem. Co.)	21 g	840 g	840 g	532 g	491 g
Sodium Hydroxide	8.4 g	330 g	330 g	312 g	288 g
Concentrated HCl	16 ml	426 ml	188 ml	307 ml	136 ml
Octanoic Acid (Matheson, Coleman & Bell)	0.1 ml	4.0 ml	4.0 ml	2.0 ml	2.0 ml
Thiodiglycol (Pierce Chem. Co.)	20 ml	200 ml	200 ml	-----	-----
Brij-35 Solution (25 g/75 ml H_2O) (Pierce Chem. Co.)	2.70 ml	108 ml	108 ml	54 ml	54 ml
Final Volume	1 L	40 L	40 L	20 L	20 L

a pH adjustment: 1 ml of 50% (w/v) NaOH or 2 ml of HCl causes a change of about 0.01 pH unit in any of the 40 L or 20 L quantities of buffer.

Amino Acid Analysis

A Phoenix Amino Acid Analyzer model K8000 (Phoenix Precision Instrument Co., Philadelphia, Pa.) is used for sample analysis to identify and quantify pool components. This analyzer is based upon the Moore and Stein analytical method (19). Cation exchange columns are used for amino acid analysis. They are a 53 x 0.9 cm column for separating acidic and neutral amino acids, and a 23 x 0.9 cm column for separating the basic amino acids and ammonia. The columns are jacketed, and a circulating water bath system provides for constant operating temperature.

Amino acid analyzers effect the separation of the amino acids by displacing them, in sequence, from these columns of ion exchange resin. This separation of amino acids is dependent on the chemical composition (the charge and side group) of the amino acid; the pH, the ionic strength and rate of flow of the eluting buffer; and the temperature of operation. Each amino acid elutes from the column in an individual and independent zone. This elution pattern is consistent for a given set of conditions.

In the analyzer, buffers are forced through the columns by positive displacement pumps working at 100 p.s.i. to allow complete analysis in a reasonable length of time. Four different sodium citrate buffers of suitable pH are prepared (Table 2) or purchased. Two separate buffers are used with automatic change from the first to the second, which has a higher pH, in each column. These buffers are prepared using double distilled H_2O. Thiodiglycol is used to minimize conversion of methionine

to methionine sulfoxides during application of sample to column; octanoic acid is used as a preservative. The pH is carefully monitored with a Beckman Zeromatic pH meter (Beckman Instruments Co., Fullerton, Cal.) to a hundredth of a pH unit. Filtered buffers are stored at room temperature in stoppered plastic carboys and are stable for several months. A buffer of pH 3.30 is initially forced through the acidic and neutral column for 180 min. An automatic switch to a buffer of pH 4.25 then occurs. Beginning the analysis at a temperature of 30°C, the temperature is similarly increased to 50°C after 30 min. To separate the more basic components of the amino acid pool, a buffer of pH 4.21 is used first; then, after 120 min, a buffer of pH 5.28 is used. The temperature change occurs at 120 min from 30°C to 50°C. Prior to using prepared concentrated buffers (Beckman), our operating conditions of pH were slightly different for the buffers used with both columns.

Regeneration, stripping residual basic amino acids from the columns, is accomplished with a 0.4 N sodium hydroxide solution containing 6.75 ml of a 50% (w/v) Brij-35 solution per liter and 1 g disodium ethylene diaminetetraacetate (Fisher Scientific Co.) per liter. The sodium hydroxide solution is forced through each column for about 30 min.

The ninhydrin color reaction is used for the quantitative analysis of each amino acid. Four liters of the reagent contain 3 liters peroxide-free methyl cellosolve (Fisher Scientific Co.), 1 liter 0.4 N sodium acetate buffer (pH 5.51 ± 0.03), 1.5 g stannous chloride (Mallinckrodt Chem. Works), and 80 g of ninhydrin (Pierce Chem. Co., Rockford, Ill.).

A standard solution (Beckman) containing 0.25 μmoles of each of the naturally occurring protein amino acids is used for identification and quantitation. Quantities are determined by the height-times-width method, the peak being multiplied by the width which is measured at half the height. For each amino acid, data is reported in μmoles/g and mole percent, both with and without ammonia included in the calculations (Results).

Radioactive Assay

In efforts to more fully understand the composition and fluctuation observed in the soluble amino acid pools, uniformly labeled casein hydrolysate-^{14}C and similarly labeled valine, arginine, and glutamic acid (New England Nuclear Corp., Boston, Mass.) are used in assays. Microplasmodia are grown in semi-defined medium for approximately 4 days, pooled in one 500 ml flask, and washed 3 times with the appropriate medium. Unless stated to the contrary, 10 ml of this well dispersed cell suspension are used with varying amounts of medium and isotope in 200 ml Erlenmeyer flasks. In these assays, radioactive amino acids and carrier amino acids are diluted in double distilled water and filter sterilized together in sterile plastic disposable filters (#245, Nalge, Rochester, N.Y.). Samples are also taken for protein determinations. An average of 1.8 mg per ml of protein is used in these assays. All procedures are performed in an ice bath unless stated otherwise.

Uptake of Casein Hydrolysate in BTC Medium

To determine the fate of radioactivity incorporated into cell

components during active growth, microplasmodia are transferred to modified BTC medium (20) after growth in semi-defined medium for 2 days. This medium contains 0.25% (w/v) dextrose and 0.25% (w/v) casein hydrolysate with 10 μ Ci of casein hydrolysate-UL-^{14}C. Microplasmodia are incubated for 48 h and then collected in one flask and washed with basal salts media. Twenty-five ml of the cell suspension are added to 15 ml non-radioactive basal salts. Two ml samples are taken at 0 time, and 5 ml samples are taken at 12 h, 24 h, 36 h, and 48 h of incubation. Samples are centrifuged in 15 ml Corex centrifuge tubes (Corning Glass Works, Corning, N.J.) for 5 min at 5 000 X g. The supernatant (medium) is decanted and saved. The cells are washed with 1 ml non-radioactive basal salts and recentrifuged under the same conditions. The supernatant is added to that previously collected. For analysis, the total volume is determined, and 0.1 ml mixed with 10 ml Cocktail A (Table 3) in glass, screw-capped counting vials (Beckman). The radioactivity is determined using a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3003). In each of these assays, individual blanks and internal standards are prepared and counted. From the data recorded, counts per min minus background values (C.P.M.), total counts per min for sample volumes (T.C.P.M.), and total disintegrations per min (T.D.P.M.) are calculated.

To extract the slime from the 5 ml samples, 6 ml reagent-grade acetone is layered on the media supernatants. The viscous slime is wound out of the solution with glass rods. To precipitate the residual particulate matter, the mixture is centrifuged at 15 000 X g for 10 min

Table 3. Cocktail A used for radioactive assay.

Compound		Concentration
Toluene	(Beckman)	666 ml
POP	(Beckman)[a]	5 g
POPOP	(Beckman)[b]	0.25 g
Triton X-100	(Beckman)	333 ml

a primary fluor: 2,5 diphenyloxazole.
b secondary fluor: 1,4-bis (2-(5-phenyloxazolyl)-benzene.

at 2°C. The slime pellet is combined with the slime previously extracted. The supernatant (media) is analyzed for radioactivity, and the particulate matter (slime) is dissolved in 10 X hyamine hydroxide (Packard Instrument Co., Inc., Downers Grove, Ill.) for 24 h at room temperature. For radioactivity analysis, 0.1 ml of the hyamine hydroxide solution is analyzed using Cocktail A.

The cells collected from each sampling are analyzed for radioactivity in the soluble amino acid pool and protein. To precipitate the protein, 1 1/2 ml of a 10% (w/v) trichloroacetic acid (Fisher Scientific Co.) solution is added to cell pellets and mixed with a Super-Mixer (Matheson Scientific Co., Houston, Tex.). The acidified suspension is left to stand in an ice bath for 30 min and then centrifuged at 15 000 X g for 10 min. The supernatant is collected. The precipitate is remixed with 1 ml of 5% (w/v) trichloroacetic acid. After standing for 30 min, they are re-centrifuged. The supernatant, with that previously collected, is analyzed for radioactivity in the acid soluble pool. The precipitated protein is dissolved in 10 X hyamine hydroxide at room temperature. Warming in a hot water bath facilitates the dissolving process. On determining the respective volumes for pool and protein solutions, 0.1 ml of each is mixed with 10 ml Cocktail A and radioactivity determined.

Uptake of Casein Hydrolysate in Basal Salts Medium

To determine the fate of the radioactivity incorporated by cells during the initial stages of sclerotization, microplasmodia are transferred to salts with 10 μ Ci of casein hydrolysate-UL-^{14}C with 0.1 ml of 0.5% (w/v)

casein hydrolysate carrier solution. Twenty-five ml of pooled 4 day-old microplasmodia are transferred to 15 ml of this medium. Uptake is monitored for 9 h until 68% of the isotope has disappeared from the medium. The cells are then washed with non-radioactive basal salts and resuspended in 15 mls of this salts solution. Two ml samples are taken for medium, pool, and protein analysis for radioactivity at 0 h, 3 h, 6 h, 9 h, and 12 h. Analyses for radioactivity contained in these fractions and in slime are also made from 5 ml samples taken at 24 h, 36 h, and 48 h.

Amino Acid Transport Saturation Experiments

Experiments attempting to saturate amino acid transport systems are done with various concentrations of valine-UL-^{14}C, arginine-UL-^{14}C, and glutamic acid-UL-^{14}C. Radioisotope and non-radioactive carrier amino acid are diluted together in double distilled water. Appropriate amounts of these dilutions are added to the prepared basal salts to give the desired concentrations of amino acid.

Initially, 2×10^{-5}M valine-UL-^{14}C uptake by sclerotizing plasmodia is monitored in basal salts and basal salts with 0.5% (w/v) dextrose, 5×10^{-4}M dinitrophenol, and 400 µg/ml cycloheximide. Cell protein, acid soluble pool and media are analyzed for radioactivity from 2 ml samples taken after 0 h, 3 h, 6 h, 9 h, 12 h, and 24 h incubation.

Seven different concentrations of valine are used to cover a wide range to attempt saturation of the transport system for this amino acid. Specifically, 2×10^{-5}M, 6×10^{-5}M, 1.2×10^{-4}M, 1.8×10^{-4}M,

2.4×10^{-4}M, 3.0×10^{-4}M, and 4.0×10^{-4}M concentrations of valine-UL-^{14}C are included in basal salts, and uptake by the cells is monitored from 2 ml samples taken at 0 h, 2 h, and 4 h intervals. Radioactivity in media and the whole cells, which are dissolved in 10 X hyamine hydroxide, is determined from 0.1 ml aliquots of the respective solutions.

Similar assays involving arginine-UL-^{14}C and glutamic acid-UL-^{14}C uptake by cells in basal salts are performed. Concentrations of 2×10^{-5}M, 1.8×10^{-4}M, 2.4×10^{-4}M, 3.0×10^{-4}M, and 4.6×10^{-4}M arginine-UL-^{14}C or glutamic acid-UL-^{14}C are included in basal salts medium to obtain possible saturation kinetics with these amino acids. At 0 h, 2 h, and 4 h, radioactivity in the media and the whole cells is determined.

Amino Acid Competition Experiments

As possible competitors, L-leucine and L-norvaline (Sigma Chem. Co.) in 15 μM quantities are included in separate flasks of basal salts containing 2×10^{-5}M valine-UL-^{14}C. After 0 h, 2 h, 4 h, 6 h, 9 h, and 12 h incubation in the medium, 2 ml samples are taken from these flasks and the radioactivity in the whole cells and the medium is determined.

Canavanine, L-lysine, and L-valine (Sigma Chem. Co.) in 15 μM concentrations are included in separate flasks containing basal salts with 2×10^{-5}M arginine-UL-^{14}C. In a similar manner, 15 μM of L-aspartic acid (Sigma Chem. Co.) is included in basal salts containing 2×10^{-5}M glutamic acid-UL-^{14}C. For both systems, radioactivity in the whole cells and the media is determined after cells have incubated for 0 h, 2 h, 4 h, 6 h, and 9 h in their respective media.

$^{14}CO_2$ Production by Catabolism of Amino Acids-^{14}C

To account for total radioactivity added to the basal salts medium and to detect possible catabolism of amino acids in the medium, potassium hydroxide (Mallinckrodt Chem. Works) is used to collect any $^{14}CO_2$ produced by the cells during incubation with valine-UL-^{14}C, arginine-UL-^{14}C, or glutamic acid-UL-^{14}C. Small tubes with 1 ml of the appropriate concentrations of KOH are suspended by wires in the respective culture flasks.

Liberation of $^{14}CO_2$ by sclerotizing plasmodia in basal salts containing 2 x 10^{-5}M valine-UL-^{14}C is measured using 20% (w/v) KOH. At 0 h, 1 h, 2h, 5 h, 7 h, 9 h, and 24 h, the KOH is removed from the culture flasks and radioactivity determined. Non-radioactive KOH is again added to the tubes. This procedure is repeated when 0.5% (w/v) dextrose is included in the same medium.

$^{14}CO_2$ is similarly collected when cells are incubated in basal salts containing 2 x 10^{-5}M and 2.4 x 10^{-4}M arginine-UL-^{14}C or glutamic acid-UL-^{14}C. After 0 h, 2 h, 4 h, 6 h, and 9 h incubation, KOH is removed and analyzed for radioactivity. Carbon dioxide collection is also made with 50% (w/v) KOH at 0 h and 9 h when cells are incubated with 1.8 x 10^{-4}, 3.0 x 10^{-4}M, and 4.6 x 10^{-4}M arginine-UL-^{14}C in basal salts. A more concentrated KOH (50%(w/v) solution is required as it is not replaced at short time intervals, but is allowed to absorb $^{14}CO_2$ for a 9 h incubation period. The same procedure is followed when these concentrations of glutamic acid-UL-^{14}C are included in basal salts. To determine

the radioactivity in the KOH, either 0.03 ml of the 20% (w/v) KOH or 0.06 ml of a 1:10 dilution of the 50% (w/v) KOH solution is mixed with 10 ml Cocktail A.

Protein Analyses

At 0 h and 48 h in their respective media, specific protein determinations are made from cells in 200 ml basal salts and in this volume of salts including one of the following (final concentrations indicated):

12.4 mM ammonium nitrate

12.4 mM ammonium chloride (J.T. Baker Chem. Co.)

12.4 mM sodium nitrate (J.T. Baker Chem. Co.)

6.2 mM ammonium nitrate

0.5% (w/v) dextrose

0.5% (w/v) casein hydrolysate

1% (v/v) DMSO.

When casein hydrolysate is included in basal salts, protein content of the cells is also determined at 96 h.

To monitor changes in protein content of sclerotizing plasmodia in basal salts solution, protein determinations are similarly made for those cells incubated for 0 h, 12 h, 24 h, 36 h, and 48 h.

For determining total protein content of microplasmodia and microsclerotia, a small aliquot of these cells is centrifuged at 14 000 X g for 5 min. Two ml of 10% (w/v) trichloroacetic acid and 2 ml of acetone are added to the cell precipitate (15), which is resuspended by vibrating on a Super-Mixer. The suspension is allowed to sit overnight at

4°C and then recentrifuged. The supernatant, containing the yellow pigment, is poured off. One more acid-acetone treatment for a few h may be necessary to completely extract the cell pigment, which interferes with the spectrophotometric analysis. One ml of 0.4 N NaOH is added to the cells for each 1 ml aliquot originally removed from culture flasks, and the cell precipitate is dissolved by heating in a hot water bath. Dilutions (1:10) are made with 0.4 N NaOH. Protein concentrations are determined by the method of Lowry, et al., using crystalline bovine serum albumin (Sigma Chem. Co.) as a standard (21, 22).

Dry Weight Determination

Dry weight determinations are made from aliquots of cells in basal salts and basal salts with 12.4 mM ammonium nitrate, 0.5% (w/v) dextrose, 0.5% (w/v) casein hydrolysate, and 1% (v/v) DMSO at 0 h and 48 h (23). Cells in basal salts with casein hydrolysate are also dried from 96 h samples. Cells are collected from a 5 min, 14 000 x g centrifugation. They are washed gently onto a pre-tarred Millipore filter (Millipore Corp., Bedford, Mass.), and a gentle vacuum is applied for about 30 sec. Care is taken not to burst the cells. The pellet of cells and filter are weighed, and the wet weight is determined. They are dried in an evenly heated (85°C) Thelco Gravity Oven (Model 17 Precision Scientific Co., Chicago, Ill.) for 36 h to 48 h until there is no further decrease in weight. At this time, the dried cells are removed from the oven and left to cool in a dessicator which contains a bed of calcium chloride pellets. Upon cooling to room temperature, the cells are quickly

weighed to avoid moisture absorption. Correction is made for filter weight, and the dry weight and percentage water are determined.

RESULTS

Morphological Observations

Sclerotization occurs when microplasmodia are incubated in a solution of basal salts in shake culture. However, as observed microscopically, the addition of casein hydrolysate, ammonium nitrate, or glutathione to the basal salts solution slows the sclerotization process (Table 4). For instance, at 48 h, basal salts-incubated cells are usually rounded, pigmentation is denser, and streaming is slow while sclerotial walls are forming (Table 4, Fig. 1C). Including casein hydrolysate in salts solution, however, results in cells that are still elongated and very actively streaming (Fig. 2A). In this same time period, ammonium nitrate or glutathione also inhibits the rate of sclerotization since the cells show active streaming, little conversion to a round shape, and little concentration of pigment (Figs. 2B, C).

In contrast to the above compounds in basal salts, are those seemingly stimulating sclerotization and those maintaining a rate comparable to cells incubated in basal salts for 48 h (Table 4). For example, aminonicotinamide addition to basal salts results in cells that are forming cell walls around the spherical cells, have relatively dense coloration, and have ceased streaming (Fig. 3A). Also, the presence of dimethyl sulfoxide stimulates sclerotization producing approximately 35% to 50% spherule organization within the sclerotia (Fig. 4A). Cells grown with dextrose and hematin show similar rate effects (Fig. 3B). In fact, these cells form berry-like spherules, without the preliminary cell rounding,

Fig. 1. Plasmodia after 48 h incubation in basal salts solutions inducive to sclerotization. Microplasmodia are cultured in growth medium (semi-defined medium) for approximately 4 days. The cells are washed with basal salts solution, and the wash is discarded. The cells are resuspended in basal salts containing the following compounds (final concentrations indicated): (A) 0.5% (w/v) dextrose (1,550x); (B) 0.2 mM C-AMP (1,440 x). The cells are also incubated in basal salts solution alone (C) (1,800x). Plasmodia are photographed with the use of a light microscope after 48 h incubation in these media.

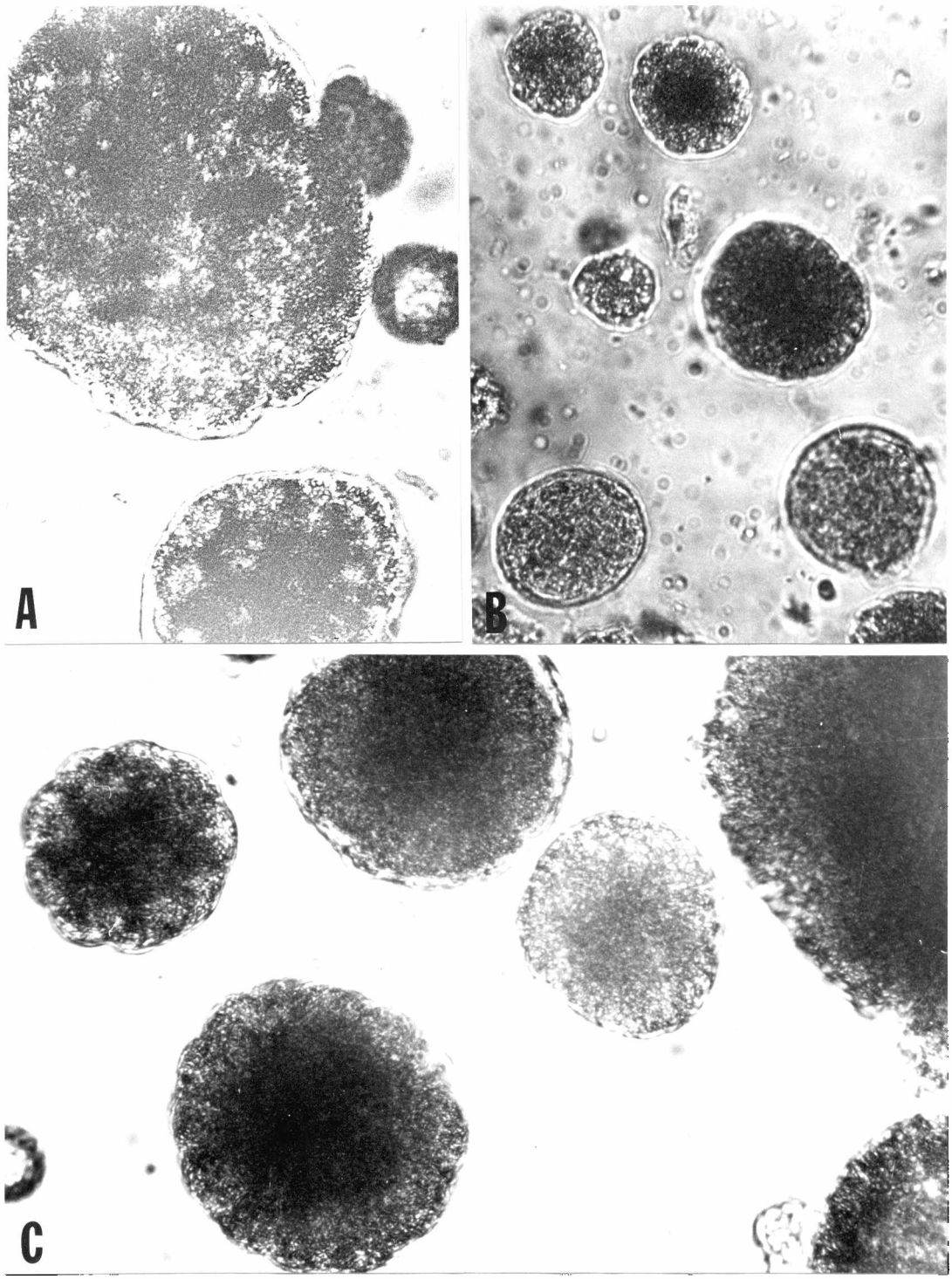

A
B
C

Fig. 2. Plasmodia after 48 h incubation in basal salts containing compounds inhibitory to sclerotization. Microplasmodia are prepared and the cells are photographed as in Figure 1. The cells are incubated in (A) basal salts containing 0.5% (w/v) casein hydrolysate (2,000x); (B) basal salts containing 12.4 mM ammonium nitrate (1,330x); and (C) basal salts containing 1 mM glutathione (1,280x).

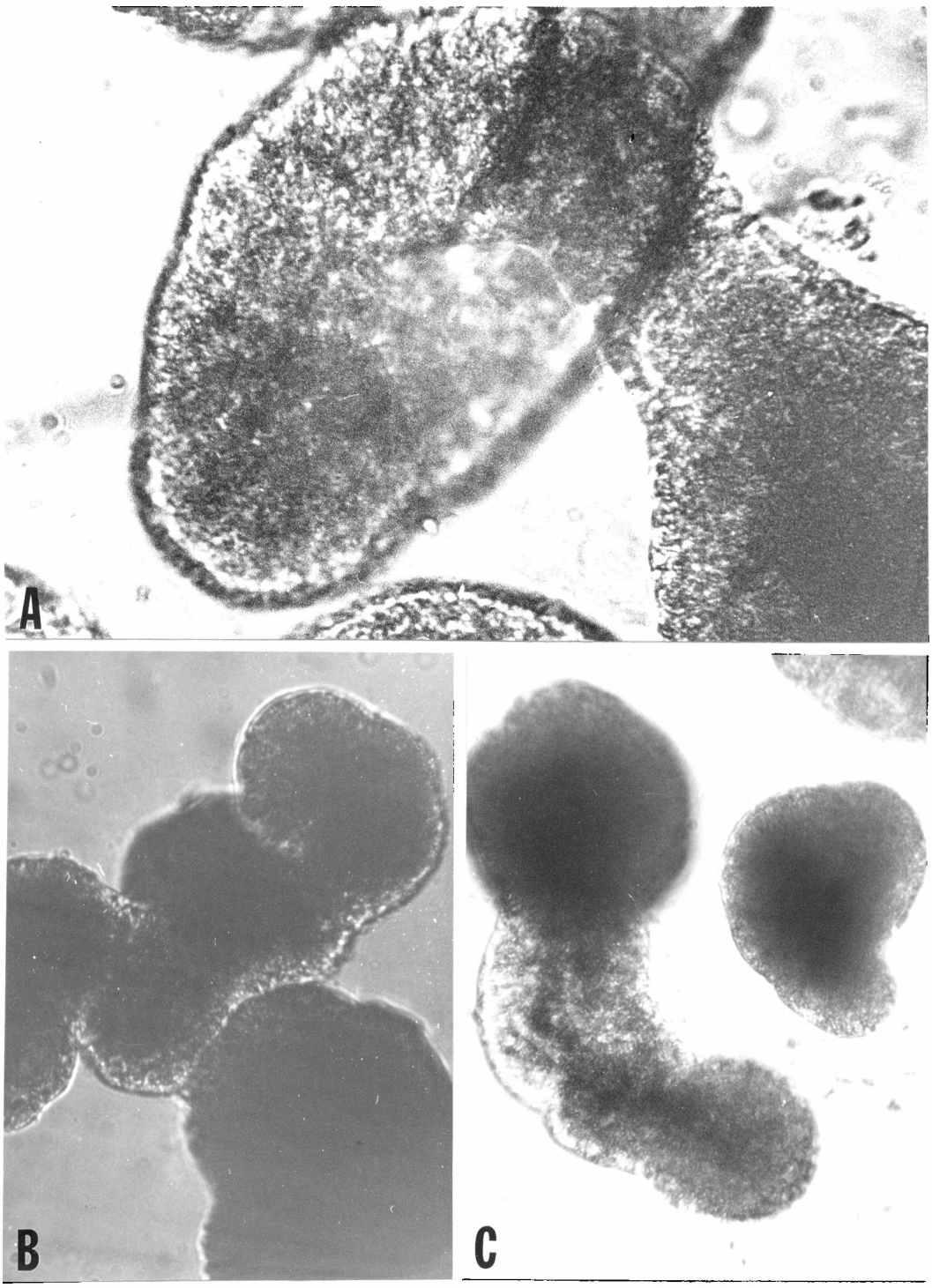

A
B
C

Fig. 3. Plasmodia after 48 h incubation in basal salts containing compounds inducing spherule formation. Experimental conditions are the same as in Figure 1. Microplasmodia are incubated in basal salts containing (A) 1mM aminonicotinamide (2,300 x); and (B) 0.5% (w/v) dextrose and 0.0025% (w/v) hematin (2,000 x).

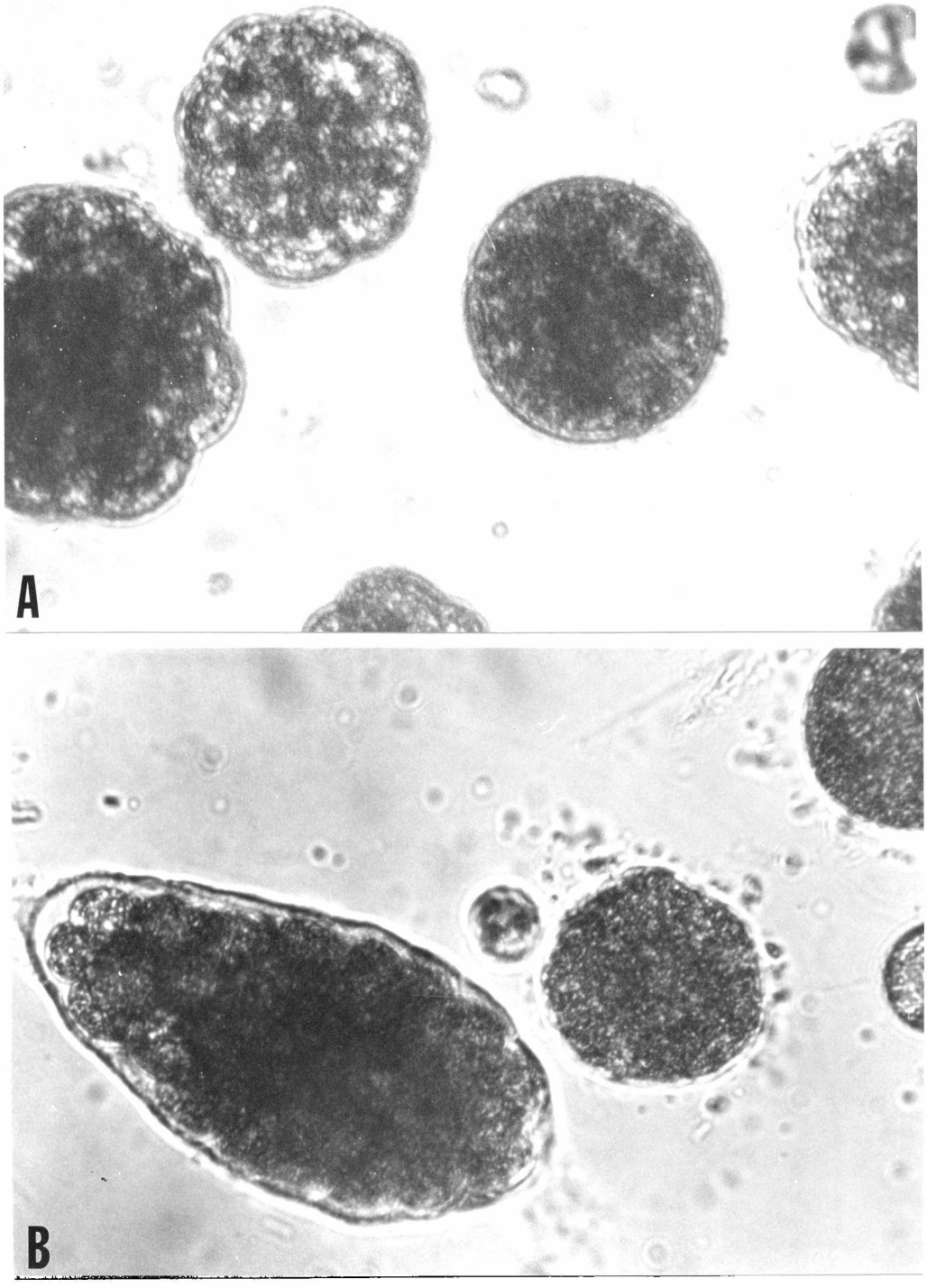

A
B

Fig. 4. Plasmodia after 48 h incubation in basal salts containing compounds inducing sclerotization. Microplasmodia are prepared and the cells are photographed as in Figure 1. These starvation media contain (A) 1% (v/v) DMSO (1,620x); (B) 0.25mM P I (1,640x); and (C) 10^{-3}M diamide (1,690 x).

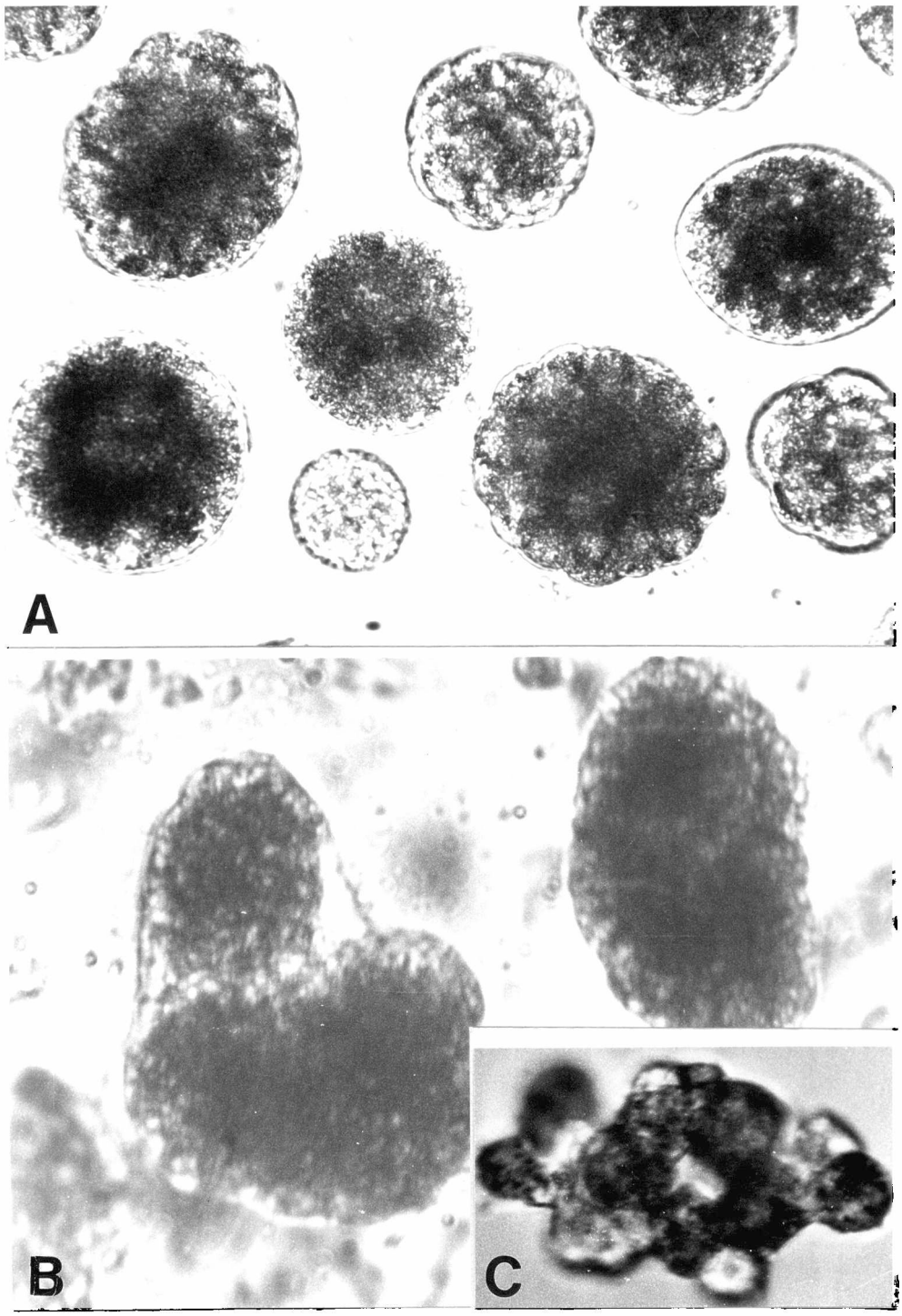

A
B
C

Table 4. Morphological observations of cells in basal salts with various compounds.

Solution	Time (Hours)	Rounding[a]	Cell Wall Formation[a]	Spherule Organization[a]	Streaming[b]	Color Intensity[c]
Basal Salts	24	5 (87%)	3-4	0	1	3
	48	5 (98%)	5	1 (12%)	2	4-5
	72	5 (100%)	5	5 (92%)	5	5
	96	5 (90%)	4-5	2-3	3-4	4-5
Casein Hydrolysate (0.5%(w/v)	24	3	2-3	0	1	1
	48	5 (86%)	4	0	1	2
	72	5 (95%)	5	1	1-2	2-3
	96	5 (100%)	5	1	1-2	2-3
Casein Hydrolysate (0.5%(w/v) & hematin (0.0025% (w/v)	24	4	2-3	0	1	1
	48	4 (75%)	4	0	1	2
	72	5	5	1	1	3
	96	5 (95%)	5	1 (15%)	1	3
Casein Hydrolysate & dextrose (0.5%(w/v) each)	24	4	2-3	0	1	1
	48	4 (75%)	4	0	1	1-2
	72	4	5	0	1	2
	96	4	5	1	1-2	2
Casein Hydrolysate (0.5%(w/v), dextrose (0.005%(w/v), hematin (0.0025%(w/v)	24	4	3	0	1	1
	48	5 (90%)	4	1	1	2
	72	5 (96%)	5	1	1	2
	96	5 (100%)	5	1	1	2
Glutathione (1 mM)	24	1	2-3	0	1	1-2
	48	2-3	4-5	0	1	2
	72	5	5	1	2	2
	96	5	5	2	2	2
Ammonium nitrate (12.4mM)	24	1	2	0	1	1
	48	3	3	0-1	1	1
	72	3	4-5	3 (55%)	3	2

Table 4, cont.

Solution	Time (Hours)	Rounding[a]	Cell Wall Formation[a]	Spherule Organization[a]	Streaming[b]	Color Intensity[c]
Ammonium chloride (12.4mM)	24	1-2	2-3	0	1	1
	48	4-5	4-5	0	1-2	1
	72	5	5	3	3	3-4
Sodium nitrate (12.4mM)	24	1-2	2	0	1	1
	48	5	4	1	2	3
	72	5	5	4	4	4
BTC medium minus hematin	24	4 (73%)	2-3	0	1	1
	48	4-5	4	0	1	1-2
	72	5	5	0	1	2
	96	5	5	1	1-2	2
Dextrose (0.5%(w/v)	24	5 (83%)	5	0	1	1
	48	5 (98%)	5	1	2	2
	72	5 (100%)	5	5 (92%)	5	3
	96	5 (92%)	4-5	5 (100%)	5	4
Dextrose (0.5%(w/v)& hematin (0.0025%(v/v)	24	4	3-4	0	1	1
	48	5 (100%)	5	1	1-2	3
	72	5	5	5 (96%)	5	4
	96	5	5	5 (100%)	5	5
DMSO (1% (v/v)	24	4 (75%)	3-4	0	1	4
	48	5	5	2 (27%)	2	5
	72	5	5	5	5	5
	96	5	4-5	1	1-2	4-5
Aminonicotinamide (1 mM)	24	5 (82%)	3-4	0	1	4
	48	5	5	1 (13%)	1	5
	72	5	5	5	5	5
	96	5	4	1	1-2	4-5
DMSO (1%(v/v) & Aminonicotinamide (1 mM)	24	5 (95%)	3-4	0	1	4
	48	5 (95%)	5	2	2	4-5
	72	5	5	5	5	5
	96	5	4	1	1-2	4
Diamide (1 mM)	24	5	4	0	1-2	3
	48	5 (100%)	4-5	2	5	4-5
	72	5	5	5	5	5

Table 4, cont.

Solution	Time (Hours)	Rounding[a]	Cell Wall Formation[a]	Spherule Organization[a]	Streaming[b]	Color Intensity[c]
C-AMP & dC-AMP (0.2 mM each)	24	5 (90%)	3-4	0	1	2-3
	48	5	4-5	1	5	2-3
	72	5	5	5	5	5
PI (0.25 mM)	24	2-3	3-4	0	1	1-2
	48	5	4-5	0	2	3
	72	5	5	0-1	4	4
Hematin (0.0025% (w/v)	24	5 (90%)	2-3	0	1	2
	48	5 (100%)	4	0	1	4-5
	72	5	5	0	1	5
	96	5	5	0-1	1	5
Deionized water	24	5 (97%)	5	0	1	4
	48	5 (100%)	5	1	2	4

a Rounding, Cell Wall Formation, Spherule Organization: 1) 0-20%; 2) 20-40%; 3) 40-60%; 4) 60-80%; 5) 80-100%.

b Streaming: (% cells having streaming) 1) 100-80%; 2) 80-60%; 3) 60-40%; 4) 40-20%; 5) 20-0%.

c Color Intensity: 1) lightest; 2) lighter; 3) color of cells in basal salts; 4) darker; 5) darkest.

and contraction of their cytoplasm (Fig. 3B). Thought to be an oxidizing agent of glutathione in some systems (24), diamide in basal salts produces cells also showing increased percentages of cell rounding, slowed streaming, and extensive cell wall formation (Fig. 4C). Less dramatic in affecting the rate of sclerotization, but nevertheless significant, is the addition of dextrose to the salts media. The cells in this media, while resembling those of basal salts in degree of sclerotization, produce clusters of many small berry-like spherules (Fig. 1A). The addition of C-AMP to basal salts arrests streaming in about 80% of the sclerotia. However, the cells in this solution are morphologically very similar to those in basal salts after the same incubation period (Fig. 1B).

In addition to these compounds and their observed effects, PI appears to be lethal for the cells when included in basal salts medium (Table 4). Those cells surviving the 48 h incubation, however, resemble those cells in basal salts in possessing contracted cytoplasm within the newly forming cell walls and slowed streaming (Fig. 4B).

After 96 h incubation in the above sclerotization solutions, the microsclerotia are transferred to semi-defined growth medium to check for viability. When the microsclerotia have been in the growth medium for approximately 24 h, they revert to the active growth state by forming microplasmodia possessing active protoplasmic streaming. Those viable cells incubated in the presence of PI remain active when transferred to growth medium.

Soluble Amino Acid Pools

When comparing the soluble amino acid pool of microplasmodia with those of microsclerotia cultured in basal salts and various other salts solutions, certain patterns manifest themselves. In plasmodia, which have reached maximum growth in semi-defined media and contain approximately 233.3 mg protein per 200 ml, sixteen of the naturally occurring protein amino acids are identified and quantified, including methionine which appears only in trace amounts. Serine, proline, and particularly valine appear in the greatest concentrations. The concentration of ammonia within the cells is also determined. On the average, 22.4 micromoles of amino acids and 5.5 micromoles of ammonia per g (wet weight) of cells appear in the soluble plasmodial pool (Tables 5 and 6).

The soluble amino acid pool of those cells sclerotizing in basal salts for 48 h resembles that of the growth stage in the case of some amino acids, yet shows striking differences in comparison to others. Fourteen amino acids are identifiable from chromatograms with methionine again present in trace amounts (Table 5). The concentrations of about seven of these amino acids remain relatively unchanged with respect to those found in the plasmodia. Two amino acids, arginine and histidine, tend to increase in concentration and five amino acids decrease. Proline, valine, and lysine decrease considerably and tyrosine and phenylalanine are not detectable in the pool. Ammonia concentration is 122% greater than that found in the plasmodia. Total pool amino acids decrease by 75% after cells have been in basal salts for 48 h (Tables 7 and 8).

Table 5. Quantitation of amino acids and ammonia in soluble pool.

Pool Component	Growing Plasmodia	Sclerotizing Plasmodia					
		Basal Salts (48 h)	Dextrose (48 h)	Ammonium Nitrate (48 h)	DMSO (48 h)	Casein Hydrolysate (48 h)	Casein Hydrolysate (96 h)
Aspartic Acid	2.0±0.3[a]	1.0±0.1	2.6±1.6	0.5±0.1	0.9±0.1	0.7±0.2	0.7±0.
Threonine	4.3±0.4	4.6±0.4	4.6±0.8	2.2±0.9	3.1±0.5	4.0±0.6	6.3±1.2
Serine	11.8±0.4	10.7±1.8	14.8±0.8	7.3±3.0	6.5±0.7	5.7±0.7	8.9±1.6
Glutamic Acid	3.5±0.6	1.3±1.2	6.8±3.0	3.2±0.6	3.1±0.5	1.9±0.2	2.3±0.2
Proline	10.0±3.8	3.2±0.0	3.2±0.1	2.6±1.2	4.2±0.9	5.1±0.1	7.2±0.
Glycine	2.4±0.3	3.2±0.1	2.5±0.1	3.6±1.5	3.2±0.4	1.5±0.0	1.9±0.4
Alanine	8.3±2.8	11.4±0.8	18.6±2.4	8.2±4.5	6.0±0.9	5.4±0.9	4.6±1.8
Valine	13.2±5.5	5.0±0.1	9.0±1.3	2.1±0.9	1.7±0.1	10.4±1.6	11.5±1.3
Methionine	Trace	Trace	0	0.1±0.1	Trace	0.4±0.0	Trace
Isoleucine	4.2±0.2	3.5±0.1	1.7±0.2	1.5±0.9	1.4±0.1	4.5±0.2	5.0±0.
Leucine	8.9±1.9	5.0±0.3	4.4±0.2	1.8±0.6	1.9±0.2	10.7±0.6	5.5±0.2
Tyrosine	0.4±0.4	0	Trace	0	Trace	0.9±0.4	0.6±0.
Phenylalanine	0.8±0.8	0	Trace	0	Trace	1.1±0.7	Trace
Ammonia	19.8±4.1	44.0±2.4	29.3±9.6	64.7±14.1	59.0±7.5	33.3±4.6	40.8±1.
Lysine	6.8±0.4	1.0±0.0	2.5±0.1	2.3±1.1	9.0±4.2	12.0±1.0	4.2±0.
Histidine	0.9±0.3	1.8±0.2	Trace	0	Trace	0.6±0.1	Trace
Arginine	2.8±0.6	4.3±0.6	Trace	Trace	0	1.6±0.8	Trace
Total moles/g(wet wt.)	27.9	10.0	3.8	10.5	4.8	16.0	16.0

a data are expressed as mean of mole percentage ± average deviation from mean of at least 2 analyses.

Table 6. Quantitation of amino acids in soluble pool.

Pool Component	Growing Plasmodia	Sclerotizing Plasmodia					
		Basal Salts (48 h)	Dextrose (48 h)	Ammonium Nitrate (48 h)	DMSO (48 h)	Casein Hydrolysate (48 h)	Casein Hydrolysate (96 h)
Aspartic Acid	2.4±0.3[a]	1.7±0.2	3.4±1.8	1.4±0.7	2.2±0.1	1.0±0.2	1.2±0.1
Threonine	5.3±0.2	8.3±0.3	6.5±0.2	6.3±0.1	7.6±0.2	6.0±0.4	10.8±2.4
Serine	14.7±1.2	19.0±2.4	21.1±1.8	20.4±0.4	16.1±1.3	8.6±0.4	14.9±2.2
Glutamic Acid	4.4±0.9	2.4±0.4	9.3±3.0	11.5±6.2	8.0±2.8	2.8±0.1	3.9±0.4
Proline	13.8±5.5	5.7±0.2	4.7±0.5	7.3±0.4	10.3±0.2	7.7±0.7	12.2±0.2
Glycine	3.0±0.6	5.8±0.2	3.6±0.4	10.2±0.2	7.9±0.6	2.2±0.2	3.3±0.5
Alanine	10.2±2.9	20.4±0.6	26.3±0.2	21.7±4.0	14.8±0.6	8.3±1.8	7.7±2.8
Valine	16.2±6.0	9.0±0.6	12.8±0.1	5.7±0.3	4.2±0.5	16.2±6.0	19.6±2.9
Methionine	Trace	Trace	0	0.4±0.4	Trace	0.7±0.0	Trace
Isoleucine	5.5±0.5	6.2±0.1	2.6±0.6	4.0±0.9	3.5±0.4	5.3±0.5	8.4±0.1
Leucine	11.0±1.8	8.9±0.1	6.3±1.2	5.1±0.3	4.6±0.4	11.0±1.8	9.2±0.6
Tyrosine	0.5±0.3	0	Trace	0	Trace	0.5±0.3	1.1±0.1
Phenylalanine	1.0±0.5	0	Trace	0	Trace	1.0±0.5	Trace
Lysine	8.5±0.9	1.8±0.2	3.6±0.4	6.1±0.7	20.8±6.5	8.5±0.9	6.5±0.2
Histidine	1.1±0.4	3.2±0.4	Trace	0	Trace	1.1±0.4	Trace
Arginine	3.5±0.6	7.7±1.4	Trace	Trace	0	3.5±0.6	Trace
Total moles/g(wet wt.)	22.4	5.6	2.7	3.5	1.9	10.6	9.5

a data are expressed as mean of mole percentage ± average deviation from mean of at least 2 analyses.

Table 7. Percent change of amino acids and ammonia during the conversion of microplasmodia to microsclerotia.

Pool Component	Percent[a]					
	Basal Salts (48 h)	Dextrose (48 h)	Ammonium Nitrate (48 h)	DMSO (48 h)	Casein Hydrolysate (48 h)	Casein Hydrolysate (96 h)
Aspartic Acid	-50	+30	-75	-55	-65	-65
Threonine	+7	+7	-49	-28	-7	+47
Serine	-9	+25	-38	-45	-52	-25
Glutamic Acid	-63	+94	-9	-11	-46	-34
Proline	-68	-68	-74	-58	-49	-28
Glycine	+33	+4	+50	+33	-38	-21
Alanine	+37	+124	-1	-28	-35	-45
Valine	-62	-31	-84	-87	-21	-13
Methionine	--	--	--	--	--	--
Isoleucine	-17	-60	-64	-67	+7	+19
Leucine	-44	-51	-80	-79	+20	-38
Tyrosine	-100	-100	-100	-100	+125	+50
Phenylalanine	-100	-100	-100	-100	+38	-100
Ammonia	+122	+48	+227	+198	+68	+106
Lysine	-85	-63	-66	+33	+76	-38
Histidine	+100	-100	-100	-100	-33	-100
Arginine	+54	-100	-100	-100	-43	-100
Total moles/g (wet wt.)	-64	-86	-62	-83	-43	-43

a data are expressed as percent increase (+) or decrease (-) in pool components when compared to growing plasmodia.

Table 8. Percent change of amino acids during the conversion of microplasmodia to microsclerotia.

Pool Component	Percent[a]					
	Basal Salts (48 h)	Dextrose (48 h)	Ammonium Nitrate (48 h)	DMSO (48 h)	Casein Hydrolysate (48 h)	Casein Hydrolysate (96 h)
Aspartic Acid	-29	+42	-42	-8	-58	-50
Threonine	+57	+22	+19	+43	+13	+104
Serine	+39	+44	+39	+10	-41	+1
Glutamic Acid	-45	+111	+161	+82	-36	-11
Proline	-59	-66	-47	-25	-44	-12
Glycine	+93	+20	+240	+163	-40	-10
Alanine	+100	+158	+113	+45	-19	-25
Valine	-44	-21	-65	-74	0	+21
Methionine	--	--	--	--	--	--
Isoleucine	+17	-51	-25	-34	0	+58
Leucine	-19	-43	-54	-58	0	-16
Tyrosine	-100	-100	-100	-100	0	+120
Phenylalanine	-100	-100	-100	-100	0	-100
Lysine	-79	-58	-28	+145	0	-23
Histidine	+191	-100	-100	-100	0	-100
Arginine	+120	-100	-100	-100	0	-100
Total moles/g (wet wt.)	-75	-88	-84	-92	-53	-58

a data are expressed as percent increase (+) or decrease (-) in pool components when compared to growing plasmodia.

Representing a "transition" between the trends of the plasmodial pool and that of microsclerotia in basal salts is the amino acid pool of cells at 48 h in basal salts containing 0.5% (w/v) casein hydrolysate (Table 5). Resembling the analysis of actively growing cells, sixteen amino acids can be identified from the pool analysis. Tyrosine and phenylalanine are again present with methionine in trace amounts. Valine is also present in amounts corresponding to that found in the plasmodial pool. Concentrations of leucine and lysine are present in increased concentrations compared to the plasmodial pool. These amino acids are also the major components in the casein hydrolysate used for incubation, comprising 26.51% and 18.13%, respectively, of the amino acids present (Table 1). Similar to the pool pattern of basal salts-incubated cells, proline has decreased in concentration in the casein hydrolysate supplemented medium. Also, ammonia concentration is 68% greater than that of the plasmodial pools and the total quantity of amino acids has decreased by 53% (Tables 7 and 8). Both values are intermediate between that of the pools of the growing stage in semi-defined media and the sclerotizing stage in basal salts (Tables 5 and 6).

Cells in basal salts and 0.5% (w/v) casein hydrolysate at 96 h also manifest pool patterns common to microsclerotia as well as plasmodia (Tables 5 and 6). In the pool, twelve amino acids are identified. Valine is found in the greatest quantity. Methionine has decreased in relation to the 48 h observation of the cell pool in the presence of casein hydrolysate. A decrease in quantity of phenylalanine resembles the observation on the

pools of cells in basal salts. Histidine and arginine are also detectable in only trace amounts, a phenomenon encountered in those cells incubated in media stimulating differentiation (0.5% (w/v) dextrose and 1% (v/v) DMSO in basal salts). Pool amino acids have decreased in concentration and, more obviously, ammonia has increased in amount when compared to the 48 h observation of cells in the presence of casein hydrolysate. Ammonia concentration has increased to amounts resembling those of cells in basal salts, and the amino acids have decreased by 58% when compared to plasmodial pools (Tables 5-8).

Inhibitory to sclerotization (as observed morphologically), ammonium nitrate in basal salts affects an ammonia concentration in the cells 227% greater than that found in the plasmodium (Table 7). Twelve amino acids are quantified from the pool with the concentration of methionine being extremely low. Relative concentrations of individual amino acids resemble those of basal salts-incubated cells. Specifically, tyrosine, phenylalanine, and histidine are lacking and arginine is present only in trace quantities in cells incubated for 48 h in basal salts containing ammonium nitrate. Total pool size decreases 84% in these soluble pools when compared to those of the plasmodia (Tables 5-8).

Observed to stimulate sclerotization, 0.5% (w/v) dextrose and 1% (v/v) DMSO in basal salts affect decreases in amino acid content of the cell pool by 51% and 66%, respectively, in comparison to the cells in basal salts medium at 48 h (Table 6). Ammonia concentration is 48% greater in cells in the presence of dextrose than in plasmodia; 198%

greater in cells in the presence of DMSO than in plasmodia (Table 7). In the cell pool from dextrose medium, glutamic acid is present in twice the quantity found in the plasmodia, and the valine concentration is intermediate between that in plasmodia and sclerotia formed in basal salts. The lysine concentration in cells incubated with DMSO is 145% greater than the amount in plasmodial pools (Table 8). In cells harvested from basal salts containing DMSO, serine, alanine, and valine also decrease in quantity in the total soluble pool when compared to sclerotia formed in basal salts or basal salts containing dextrose. Nevertheless, although these fluctuations in pool components do occur, the overall distribution of amino acids in the cells in the media stimulating sclerotization resembles that found in cells from basal salts (Tables 5 and 6).

Certain trends, therefore, become apparent from the pool analyses. Amino acid pools in cells from salts media inhibitory (determined morphologically) to sclerotization , resemble those of the vegetative state. The characteristics of these pools include high total amino acid concentration and relatively low percentages of ammonia. Compounds stimulating differentiation seem to effect higher ammonia percentages and low quantities of amino acids in the cellular amino acid pool.

Small quantities of unidentifiable amino acids are also found on chromatograms of some of the above described soluble pools. They are not of the twenty naturally occurring protein amino acids. These amino acids are not present in concentrations warranting identification.

Fate of Protein During Sclerotization in Basal Salts

To monitor protein turnover during sclerotization, cells are transferred to basal salts and aliquots taken at 0 h, 12 h, 24 h, and 48 h (Table 9). From 0 h to 12 h in basal salts, there is an insignificant decrease in protein in these cells. Possibly, protein may be continuously degraded and resynthesized or little protein decomposition may be occurring during this period of incubation. The greatest decrease of cell protein occurs from 12 h to 24 h in the salts medium. Protein concentration then steadily decreases in quantity to 48 h.

Effects of Apparent Inhibitory and Stimulatory Sclerotization Media on Protein Degradation

Cells in basal salts and basal salts containing 12.4 mM ammonium nitrate, 12.4 mM ammonium chloride, 6.2 mM ammonium nitrate, 0.5% (w/v) casein hydrolysate, 0.5% (w/v) dextrose, 1% (v/v) DMSO, and 12.4 mM sodium nitrate are analyzed for protein content at 0 h and after 48 h incubation (Table 10). After 96 h incubation in the casein hydrolysate medium, cells are also examined for protein concentration. The protein in these cells in basal salts with 0.5% (w/v) casein hydrolysate decreases the least after 48 h incubation; but after 96 h, there is a 37% decrease in protein. This represents approximately 1.5 times the degradation observed after 48 h incubation. Ammonium nitrate and ammonium chloride in concentrations of 12.4 mM produce an effect similar to casein hydrolysate with 72-75% of the original protein remaining in the cells after 48 h incubation. However, sodium nitrate (12.4 mM) added to basal salts

Table 9. Fate of cellular protein during sclerotization in basal salts.

Period of Incubation (Hours)	Decrease in Protein Concentration (%)
0	0
12	1.6
24	25.6
48	43

produces a decrease in cellular protein similar to basal salts alone. The ammonium ion, rather than the nitrate ion, is then responsible for inhibiting protein degradation during sclerotization. In addition, protein hydrolysis is 1.6 times greater in cells incubated in basal salts containing 6.2 mM ammonium nitrate at 48 h than in cells in salts with 12.4 mM ammonium nitrate (Table 10).

Protein degradation in those cells incubated in media stimulating sclerotization is greater than under the above conditions. Cells in basal salts, salts with DMSO, and salts with dextrose at 48 h show a protein decrease almost twice as great as cells in the salts medium supplemented with casein hydrolysate or ammonium compounds.

Dry Weight Determination

Dry weight determinations are made from aliquots of cells at 0 h and 48 h in basal salts and basal salts containing ammonium nitrate, casein hydrolysate, dextrose, and DMSO. From the 0 h sample, the plasmodial cells are found to contain approximately 96% water. At 48 h in their respective salts solutions, sclerotizing plasmodia consist uniformly of 93% water indicating that there is little loss of water during the conversion to sclerotia.

Uptake of Casein Hydrolysate in BTC Medium

After growth in semi-defined medium for 2 days, plasmodia are transferred to BTC medium containing casein hydrolysate-UL-^{14}C for a 48 h incubation. The cells are then suspended in non-radioactive salts

Table 10. Fate of cellular protein after 48 h incubation in basal salts containing supplements.

Additions to Basal Salts Solutions	Decrease in Protein Concentration in 48 H (%)
Basal salts, only	43
6.2 mM ammonium nitrate	45
12.4 mM ammonium nitrate	28
12.4 mM ammonium chloride	25
12.4 mM sodium nitrate	47
0.5% (w/v) casein hydrolysate	22, (37)[a]
0.5% (w/v) dextrose	50
1% (v/v) DMSO	44

a after 96 h incubation.

solution.

Of the total DPM present in the cells at 0 h, 92% is incorporated into protein and 8% into the soluble amino acid pool. At each sample interval, the majority of the cellular radioactivity is always contained in the protein fraction. Of the DPM in cells at 48 h, 92% resides in this fraction. The pool contains a relatively low but constant percentage (7%) of the DPM in the cells at each interval.

From 0 h to 48 h in basal salts, certain trends become apparent. There is a small return of radioactivity to the media (Table 11, Fig. 5) and minute amounts of radioactive slime are secreted at each sampling interval (Table 11). There is a steady decrease in radioactivity contained in the cells. Specifically, protein and pool show a steady decline during the 48 h sclerotization process in basal salts (Fig. 5). After 48 h, 58% of the radioactive protein and 60% of the radioactivity in the soluble pool have been lost (Table 12). Radioactivity is also found to be evolved as $^{14}CO_2$, and the amount of $^{14}CO_2$ produced and liberated from the sclerotizing cells is seen to steadily increase with incubation time (Table 11).

Uptake of Casein Hydrolysate in Basal Salts Medium

Plasmodia are grown in semi-defined media for 4 to 5 days and then transferred to basal salts media containing casein hydrolysate-UL-^{14}C. Uptake is monitored for 9 h in the labeled salts solution until 68% of the initial DPM has disappeared from the media. Incorporation into pool and protein is observed to steadily increase during this time interval (Fig. 6).

Fig. 5: Fate of radioactivity, incorporated into the pool and protein in growing conditions, during sclerotization in basal salts medium. Microplasmodia are grown in semi-defined media for two days and then transferred to BTC medium containing 10 microcuries casein hydrolysate-UL-^{14}C. Microplasmodia are shaken in the BTC medium for 48 h. After washing the cells, they are then suspended in "cold" basal salts solution. Sclerotizing plasmodia (1.42 mg protein/ml) are analyzed for radioactivity present in the soluble amino acid pool and protein at 12 h intervals. Radioactivity present in the medium is also determined. Protein: triangles; amino acid pool: closed hexagons; medium: open hexagons.

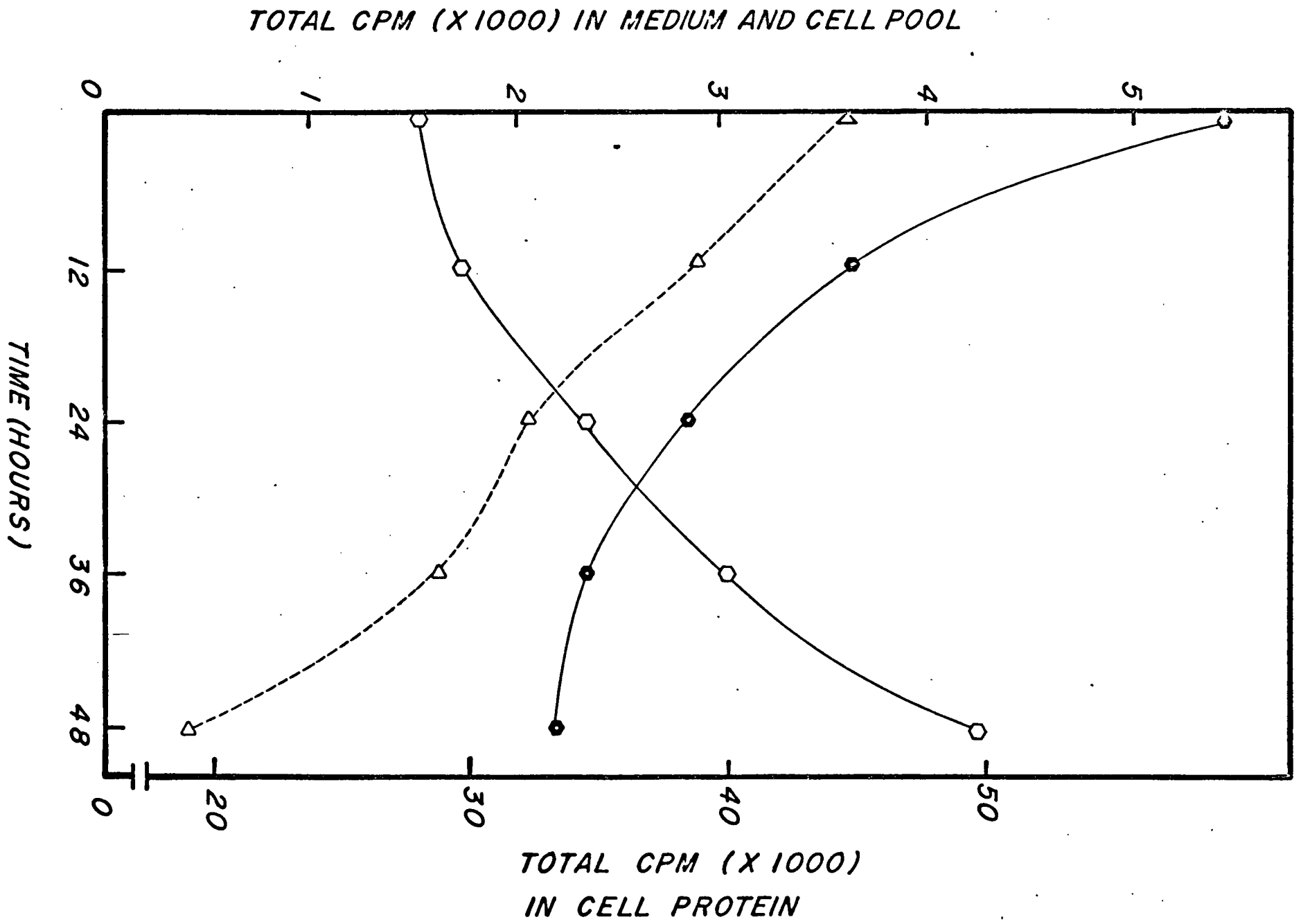

TOTAL CPM (X 1000) IN MEDIUM AND CELL POOL
0
1
2
3
4
5
TIME (HOURS)
12
24
36
48
0
20
30
40
50
TOTAL CPM (X 1000)
IN CELL PROTEIN

Fig. 6: Fate of radioactivity incorporated by cells during the first nine hours in basal salts solution. Microplasmodia are grown for 4 days in semi-defined medium. Presettled cells are washed and then suspended in basal salts containing 10 microcuries casein hydrolysate-UL-^{14}C. Incorporation is followed for 9 h until 68% of the DPM present in the medium at 0 h has disappeared from the salts solution. Sclerotizing plasmodia (1.02 mg protein/ml) are then washed and transferred to non-radioactive basal salts solution. The soluble pool and protein are analyzed for radioactivity at 12 h intervals. Cell amino acid pool: open circle; protein: closed circle.

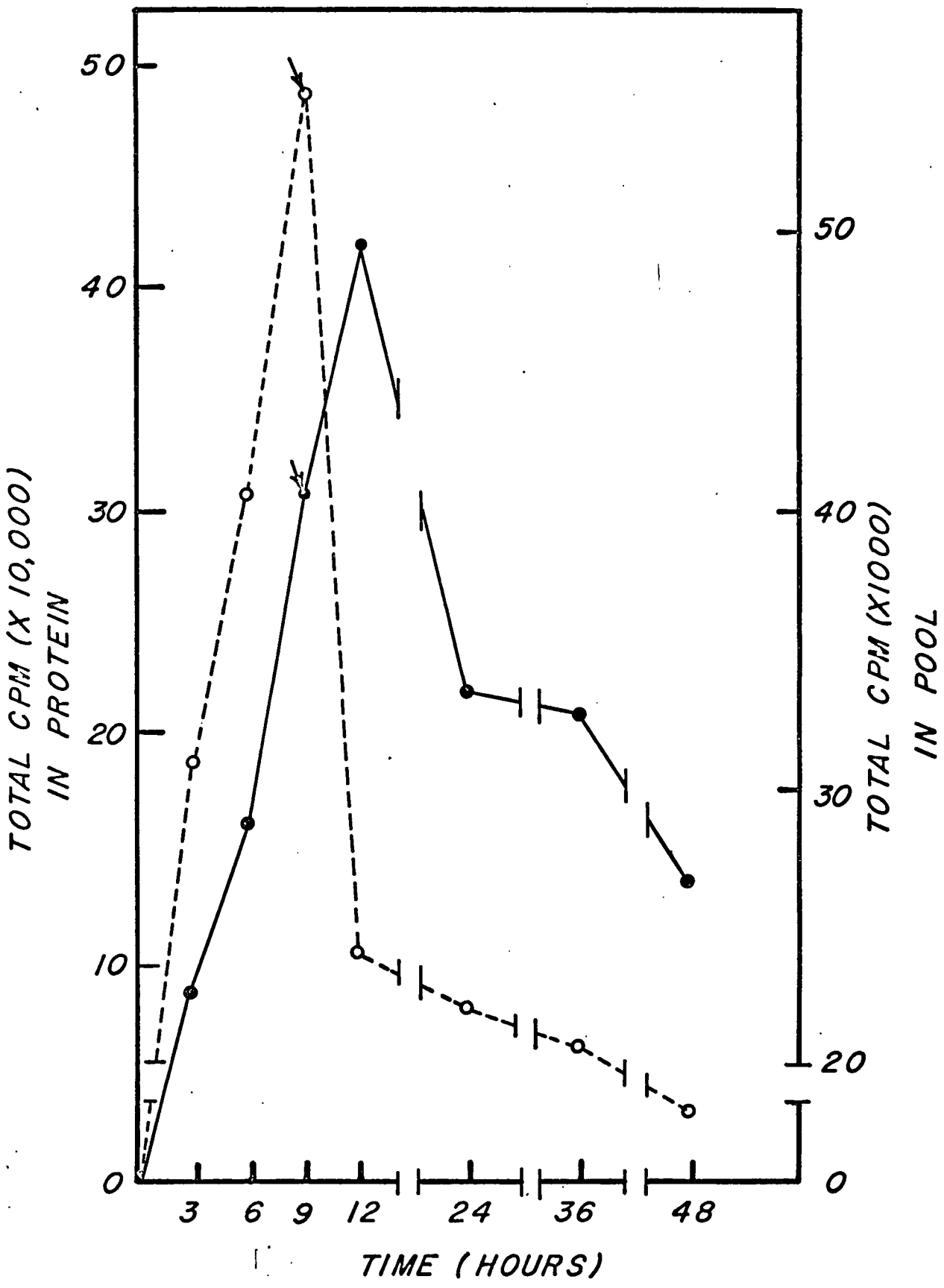

50
40
30
20
10
0
TOTAL CPM (X 10,000) IN PROTEIN
50
40
30
20
0
TOTAL CPM (X1000) IN POOL
3
6
9
12
24
36
48
TIME (HOURS)

Table 11. Fate of casein hydrolysate-UL-^{14}C incorporated by microplasmodia in BTC medium after transfer to basal salts solution.

Time (Hours)	Medium (%)[a]	Carbon Dioxide (%)[a]	Slime (%)[a]
0	0	0	0
12	3	24	2
24	3	26	2
36	5	32	2
48	7	45	3

a percent of radioactivity present in cells at 0 h (total cellular DPM taken as 100%).

Table 12. Fate of protein and pool labeled in BTC medium containing casein hydrolysate-UL-^{14}C after transfer to basal salts solution.

Time (Hours)	Protein (%)[a]	Pool (%)[a]
0	100[b]	100[c]
12	87	66
24	78	51
36	67	43
48	42	40

a percent of original DPM remaining in each fraction.

b amount of radioactivity in protein after 48 h incubation in BTC medium is taken as 100%.

c amount of radioactivity in soluble amino acid pool after 48 h incubation in BTC medium is taken as 100%.

The cells are then resuspended in a "cold" salts solution.

Of the total DPM present in the cells at 9 h, 94% resides in the cell protein and 6% resides in the amino acid pool. At each subsequent sample interval, the majority of the DPM in the cells remains in the cell protein. Of the DPM in the cells at 48 h, 92% is contained in this cell fraction. The amount of radioactivity in the amino acid pool consistently accounts for approximately 6% of the DPM in the cells at each sample interval.

Pool incorporation is maximum at 9 h in the basal salts containing casein hydrolysate and, after transfer to "cold" medium, shows a sharp decrease (Fig. 6). From 0 h to 12 h in this non-radioactive salts media, 56% of the pool radioactivity disappears (Table 13). This decrease in the soluble pool could account for the continued incorporation of radioactivity into protein during that time interval. Radioactivity in protein increased 26% from 0 h to 12 h in basal salts (Table 13). Protein incorporation peaks at 12 h and then steadily decreases (Fig. 6). Approximately 1% of the radioactivity determined to be in the cells at 0 h in the "cold" basal salts solution is found to be returned to the media during subsequent incubation. On determining the amounts of radioactivity recovered at different sample times, carbon dioxide is also found to be liberated. After 48 h in basal salts, 50% of the DPM present in the cells at 0 h can be attributed to $^{14}CO_2$ production.

Valine-^{14}C Transport and Incorporation

Valine-UL-^{14}C uptake and incorporation occurs quite readily in

Table 13. Fate of protein and pool labeled in basal salts medium containing casein hydrolysate-UL-^{14}C after transfer to non-radioactive basal salts.

Time (Hours)[a]	Protein (%)[b]	Amino Acid Pool (%)[c]
0	74	100
12	100	44
24	63	39
36	49	36
48	32	33

a 0 h represents time of transfer to non-radioactive basal salts.

b DPM present in protein at 12 h taken as 100%.

c DPM present in pool at 0 h taken as 100%.

basal salts and in salts with 0.5% (w/v) dextrose containing 2 x 10^{-5}M quantities of the amino acid (Fig. 7). When microplasmodia are transferred to the basal salts media (1.3 mg protein/ml), 20% of the labeled valine is removed from the media in 2 h. After 6 h in basal salts, 56% of the isotope is removed. The rate of valine uptake is less in the presence of glucose, since only 14% of the initial DPM present in the media disappears from the salts with 0.5% (w/v) dextrose in 2 h. Data obtained from cellular protein and pool analyses reveal incorporation of radioactivity paralleling its removal from the medium. However, more valine-UL-^{14}C is incorporated in the presence of glucose than in basal salts alone (Fig. 7).

Valine-UL-^{14}C incorporation into the soluble pool is maximum at 3 h and then steadily declines in the basal salts solution (Fig. 7). Protein incorporation is maximum at 9 h (Fig. 7). Labeled pool components could be contributing to protein incorporation from 3 h to 9 h. Similarly, when cells are incubated in basal salts containing 0.5% (w/v) dextrose, maximum incorporation of radioactivity into the amino acid pool occurs at 6 h followed by a steady decline in this fraction (Fig. 7). In this medium, protein incorporation is also maximum at 12 h (Fig. 7).

With cycloheximide and dinitrophenol included in the salts media, valine uptake is substantially decreased (Fig. 8). Microscopic examination of cells in these media after 48 h reveal bleached cells, no streaming, and apparent death. Some bleached cells are detected as early as 9 h.

Carbon dioxide production from cells in both salts and salts with

Fig. 7: Incorporation of valine-UL-^{14}C into cell pool and protein in basal salts and basal salts with glucose. Four-day-old microplasmodia are suspended in basal salts solution and basal salts containing 0.5% (w/v) dextrose. These solutions also contain 2 microcuries valine-UL-^{14}C and 2 x 10^{-5}M valine. Sclerotizing plasmodia (1.3 mg protein/ml) are analyzed for uptake of the labeled amino acid. Amino acid pool and protein are specifically extracted and analyzed for radioactivity. Basal salts pool: broken line; basal salts protein: open circle; basal salts with dextrose pool: triangle; basal salts with dextrose protein: closed circle.

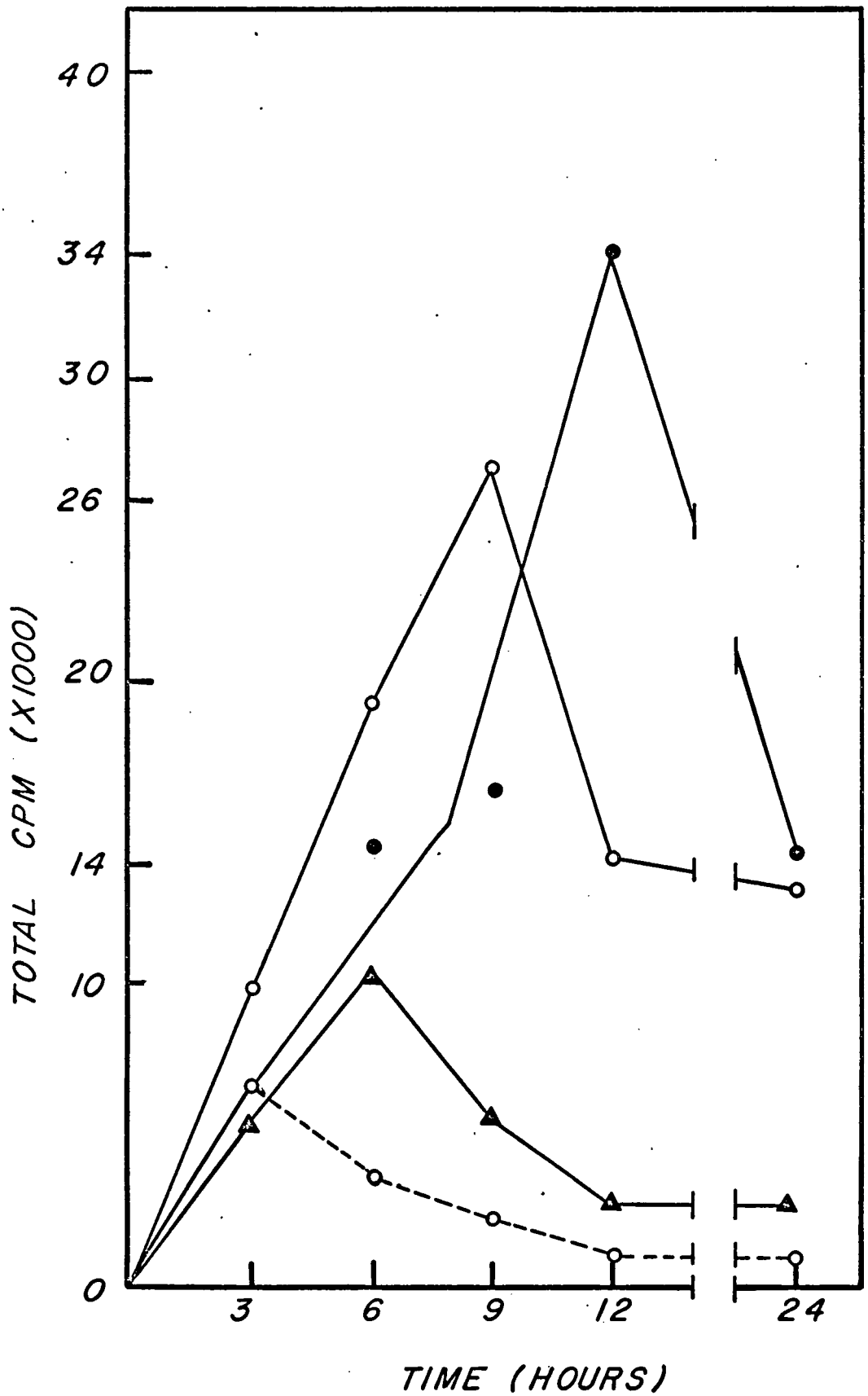
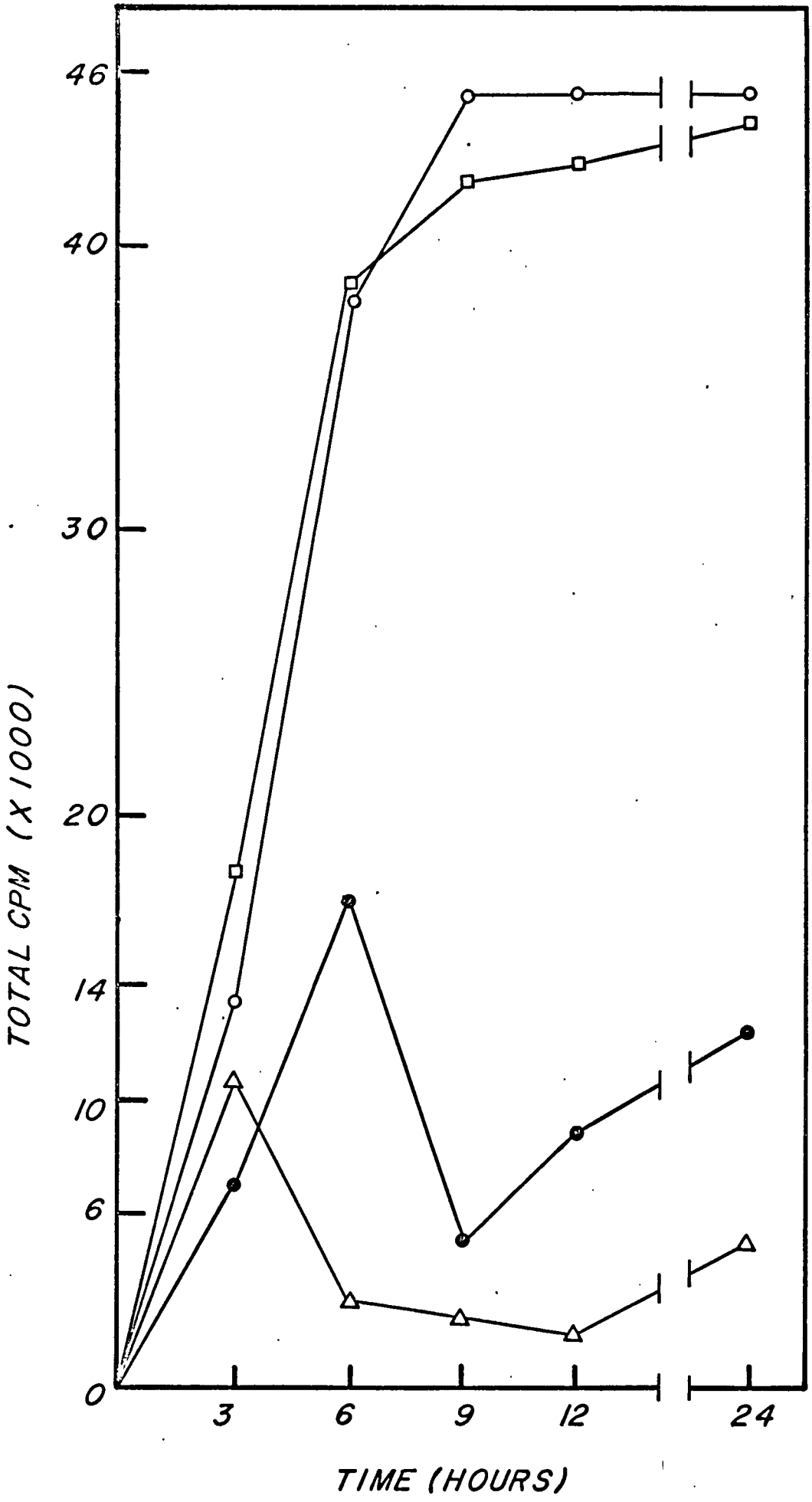

40
34
30
26
20
14
10
0
TOTAL CPM (X1000)
3
6
9
12
24
TIME (HOURS)

Fig. 8: Valine-UL-^{14}C incorporation by sclerotizing microplasmodia in basal salts solution and basal salts solution with glucose. Microplasmodia (1.3 mg protein/ml) are treated as in Fig. 7. In one flask of basal salts, 5×10^{-4}M dinitrophenol is added; to another flask of salts 400 mg/ml cycloheximide is added. All salts solutions contain 2 microcuries valine-UL-^{14}C and 2×10^{-5}M valine. Uptake of labeled amino acid is followed as described in Fig. 5. Basal salts: open square; basal salts and dextrose: open circle; basal salts and cycloheximide: closed circle; basal salts and dinitrophenol: triangle.


46
40
30
20
14
10
6
0
TOTAL CPM (X 1000)
3
6
9
12
24
TIME (HOURS)

0.5% (w/v) dextrose (1.98 mg protein/ml) is also monitored. At this protein concentration, 31% of the isotope is removed from the basal salts media in 2 h. Only 24% of the 0 h DPM disappears from the salts medium containing dextrose in this same time period. From the initial 1 h and 2 h collections, little carbon dioxide is liberated from cells in the basal salts solution or in salts with dextrose. Cumulative data from subsequent samples depict a steady increase in carbon dioxide production (Fig. 9). In each respective case, interval sample analyses also reveal this 2 h "lag" with subsequent progressively increasing amounts of liberated $^{14}CO_2$ (Table 14).

Arginine Incorporation

Arginine-UL-^{14}C is incorporated into sclerotizing microplasmodia at a rapid rate when included in basal salts containing 2 x 10^{-5}M arginine. When microplasmodia (1.34 mg protein/ml) are suspended in radioactive arginine-salts medium, 49% of the DPM present in the media at 0 h is taken up by the cells in 6 h. Analysis for the amount of radioactivity liberated as $^{14}CO_2$ shows a 2 h "lag" in which little apparent $^{14}CO_2$ is liberated by the cells. However, subsequent analyses reveal an increased production of $^{14}CO_2$ as incubation continues (Fig. 10). For instance, after 9 h incubation in the salts medium, cells release 11% of the DPM present in the media at 0 h as $^{14}CO_2$.

Glutamic Acid Incorporation

Sclerotizing microplasmodia (1.34 mg protein/ml) incorporate 70% of the DPM present at 0 h after 6 h incubation in the basal salts solution

Fig. 9: $^{14}CO_2$ liberated by sclerotizing microplasmodia in basal salts solution and basal salts with glucose containing valine-UL-^{14}C. Four-day-old microplasmodia are suspended in basal salts solution and basal salts with 0.5% (w/v) dextrose. These solutions contain 0.38 microcuries valine-UL-^{14}C and 2 x 10^{-5}M valine. Potassium hydroxide is used to absorb liberated $^{14}CO_2$ produced by sclerotizing plasmodia (1.98 mg protein/ml). Cumulative data are presented. Basal salts: open circle; basal salts with glucose: closed circle.

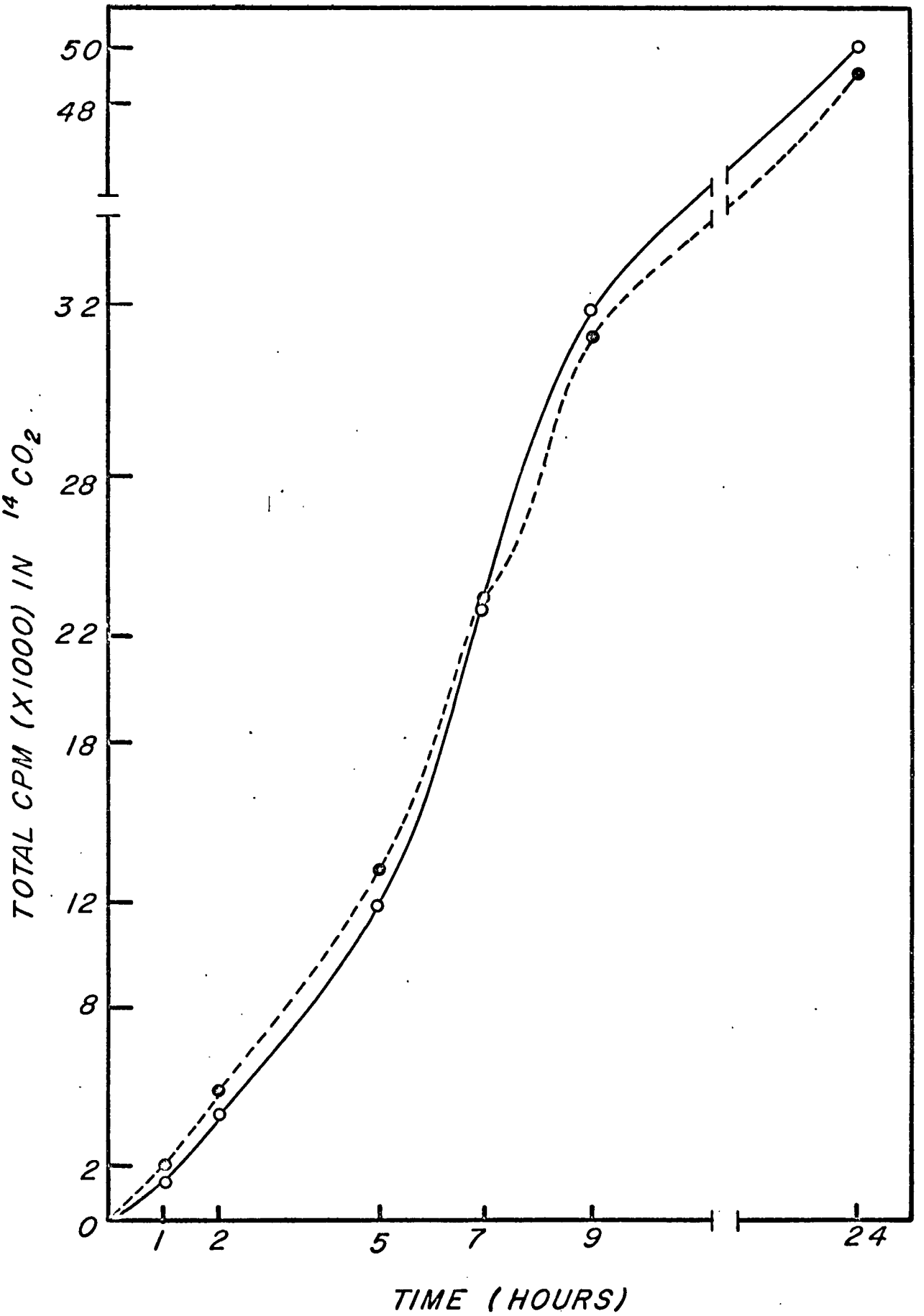

50
48
32
28
22
18
12
8
2
0
1
2
5
7
9
24
TOTAL CPM (X1000) IN $^{14}CO_2$
TIME (HOURS)

Fig. 10: $^{14}CO_2$ liberated by sclerotizing plasmodia in basal salts containing arginine-UL-^{14}C and glutamic acid-UL-^{14}C. Microplasmodia are grown in semi-defined media for 4 days, washed, and then re-suspended in basal salts. Microplasmodia (1.34 mg protein/ml) are incubated in the salts containing 0.375 microcuries of either glutamic acid-UL-^{14}C or arginine-UL-^{14}C and 2 x 10^{-5}M of the non-radioactive amino acid. Potassium hydroxide is used to absorb $^{14}CO_2$ produced by the incubated cells and cumulative data are presented. Arginine-UL-^{14}C: closed circle; glutamic acid-UL-^{14}C: open circle.

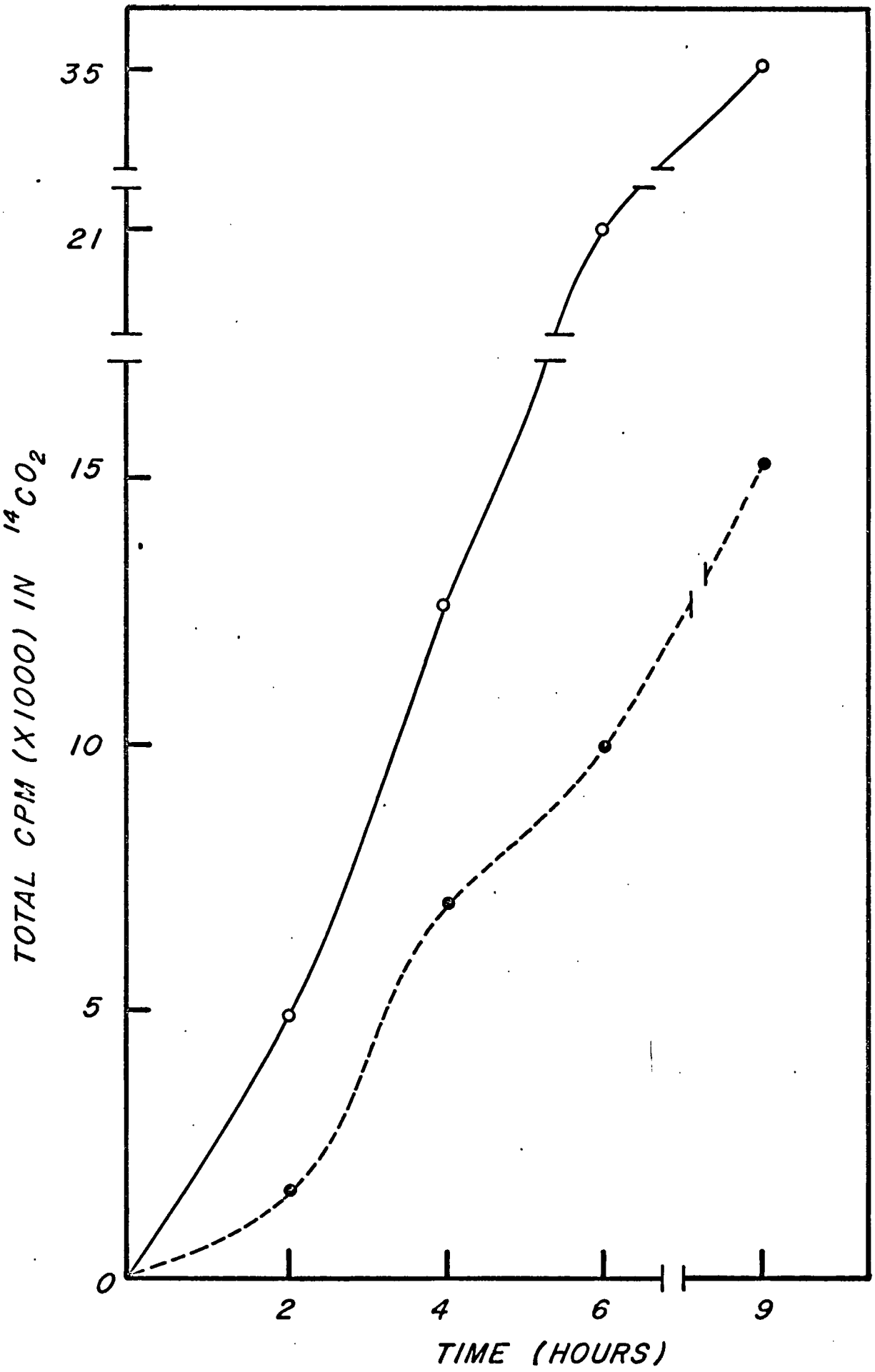

35
21
15
10
5
0
2
4
6
9
TOTAL CPM (X1000) IN $^{14}CO_2$
TIME (HOURS)

Table 14. $^{14}CO_2$ liberated by cells in basal salts and basal salts with glucose containing valine-UL-^{14}C.

Time (Hours)	Basal Salts (%)[a]	Basal Salts with Dextrose (%)[a]
0	0	0
1	3	2
2	4	3
5	8	11
7	13	14
9	14	14
24	17	15

a percentages are of total DPM present in the medium at 0 h.

containing 2 x 10^{-5}M glutamate. Of the three amino acids whose cellular transport is analyzed in basal salts, glutamic acid, then, appears to be removed from the media at the fastest rate. $^{14}CO_2$ is also produced by sclerotizing microplasmodia incubated in basal salts with this amino acid. When the production of $^{14}CO_2$ is graphed, there is a slight increase in slope at 2 h (Fig. 10). In fact, 34% of the total DPM present in the media at 0 h is recovered as $^{14}CO_2$ after 9 h incubation of the cells.

Amino Acid Competition Assays

Reported competitors for valine, arginine, and glutamic acid in other systems are included with these amino acids in basal salts solutions. Valine, arginine, and glutamic acid are present in concentrations of 2 x 10^{-5}M with their respective competitors present in concentrations of 15 μM.

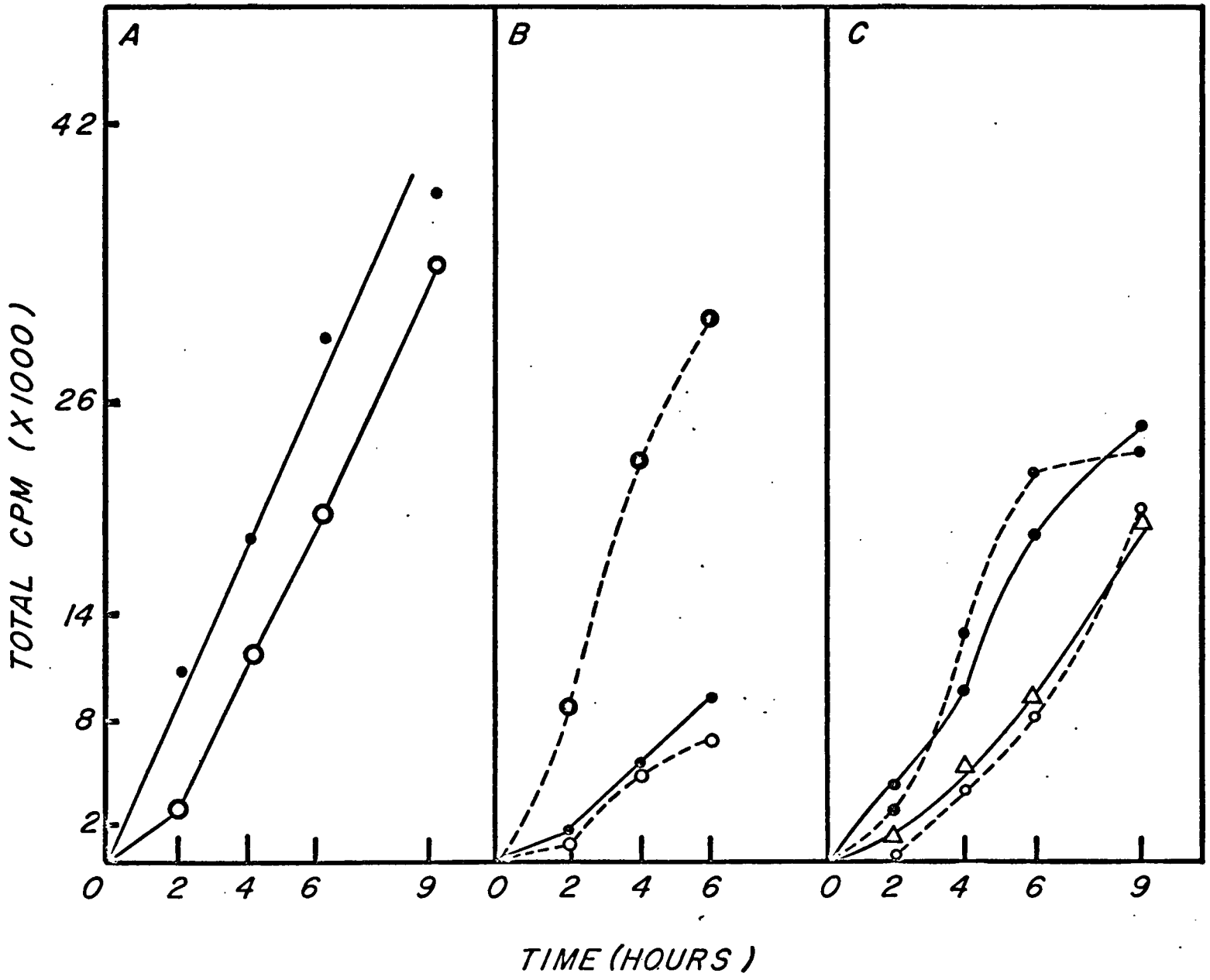
Aspartic acid appears effective in inhibiting glutamic acid incorporation by microplasmodia. After 6 h incubation in medium containing both aspartic acid and glutamic acid, microplasmodia incorporate 30% less glutamic acid than is observed when this amino acid alone is present in the incubation medium (Fig. 11A).

Similarly, L-leucine and L-norvaline appear effective in decreasing valine uptake when included in the salts medium (Fig. 11B). For example, after 6 h incubation, L-leucine affects an 81% decrease in valine uptake by the cells. L-norvaline in the medium also causes a 68% decrease in incorporation of valine into the cells in this same time period.

Canavanine, an antimetabolite, and L-lysine are equally interesting

Fig. 11: Effect of competitors on incorporation of valine, arginine, or glutamic acid by sclerotizing microplasmodia in basal salts solution. (a) Four-day-old microplasmodia are transferred to basal salts solution containing 0.38 microcuries glutamic acid-UL-^{14}C and 2 x 10^{-5}M glutamic acid. In one flask of salts, 15 micromolar L-aspartic acid is included with glutamic acid. Cells (1.34 mg protein/ml) are analyzed for incorporation of the radioactive amino acid. Uptake is followed by disappearance of radioactivity from the media. Glutamic acid-UL-^{14}C: closed circle; glutamic acid-UL-^{14}C and aspartic acid: open circle. (b) Microplasmodia (2.0 mg protein/ml) are washed, and suspended in basal salts containing 0.36 microcuries valine-UL-^{14}C and 2 x 10^{-5}M valine. To one flask, 15 micromolar L-leucine is added; to another, fifteen micromolar L-norvaline is added. Uptake of labeled valine by sclerotizing plasmodia is monitored as above. Valine-UL-^{14}C: broken line; valine-UL-^{14}C and L-leucine: open circle; valine-UL-^{14}C and L-norvaline: solid line. (c) Microplasmodia (1.34 mg protein/ml) are transferred from semi-defined medium to basal salts containing 0.38 microcuries arginine-UL-^{14}C and 2 x 10^{-5}M L-arginine. To one flask, 15 micromolar canavanine is added; to another flask, 15 micromolar L-lysine is added; and 15 micromolar L-valine is also included in another flask of salts solution. Cellular incorporation of labeled amino acid is then monitored as above. Arginine-UL-^{14}C: broken line, closed

circle; arginine-UL-^{14}C and canavanine: triangle; arginine-UL-^{14}C and L-lysine: open circle; arginine-UL-^{14}C and L-valine: closed circle, solid line.


A
B
C
42
26
14
8
2
TOTAL CPM (X1000)
0
2
4
6
9
0
2
4
6
0
2
4
6
9
TIME (HOURS)

in their inhibition of arginine uptake by microplasmodia when included with the amino acid in basal salts media (Fig. 11C). With canavanine, a 58% decrease in uptake of arginine is observed in 6 h. In medium containing L-lysine, arginine incorporation at 6 h is 62% less than is observed when the competitor is absent. However, valine does not affect the transport of arginine since arginine uptake parallels that of arginine alone in the salts medium (Fig. 11C).

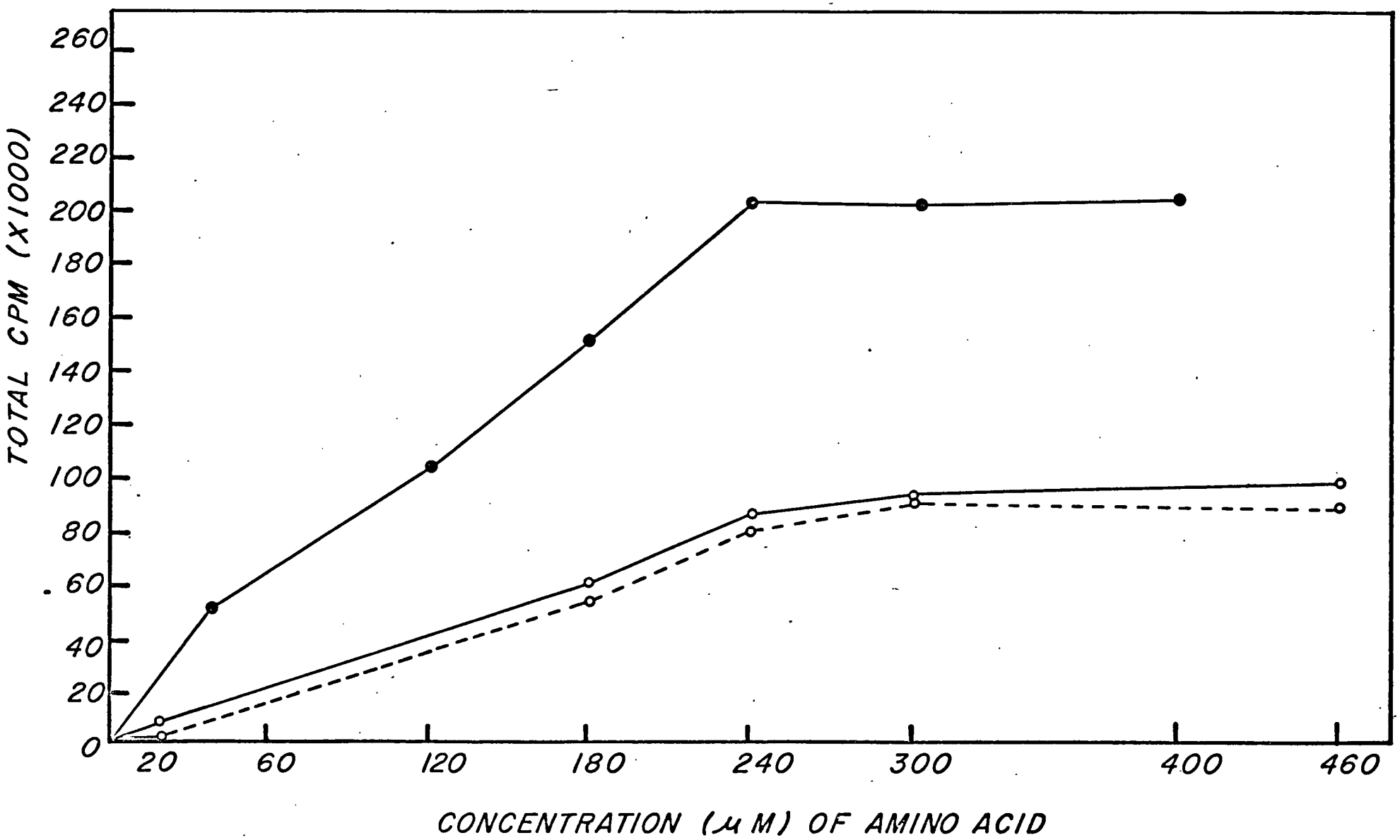
Amino Acid Transport Saturation Assays

To provide further proof for the presence of amino acid transport systems in this Myxomycete, attempts are made to achieve saturation kinetics using specific amino acids. Arginine, valine, and glutamic acid are respectively used in various concentrations in the basal salts solution.

Both 2 h and 4 h observations reveal saturation of the transport systems occurring between 2.4×10^{-4}M and 3.0×10^{-4}M concentrations of arginine and glutamic acid, respectively (Fig. 12). $^{14}CO_2$ is also collected from cells incubated with arginine or glutamic acid in basal salts solutions in concentrations used for saturation kinetics. Analyses at 9 h at each of these concentrations reveal a steady increase in amount of $^{14}CO_2$ liberated by the cells as the concentration of the amino acid increases to 2.4×10^{-4}M, at which point the amounts of $^{14}CO_2$ produced are constant.

Saturation of the transport system responsible for valine incorporation is also achieved. When this amino acid is included in basal salts in a suitable range of concentrations, uptake also levels off at 2.4×10^{-4}M valine (Fig. 12).

Fig. 12: Saturation of transport systems of valine, arginine, and glutamic acid in basal salts solutions. Microplasmodia (1.34 mg protein/ml), after growth in culture medium for four days, are suspended in basal salts containing L-arginine or L-glutamic acid. The salts solutions contain increasing quantities of the respective amino acid-UL-^{14}C. Amino acid concentrations in separate flasks are progressively increased. Uptake of the labeled amino acids is determined by the disappearance of radioactivity from the incubation medium after 2 h incubation. Four-day-old microplasmodia (3.4 mg protein/ml) are washed and also suspended in basal salts containing L-valine. The salts solutions contain appropriate amounts of valine-UL-^{14}C and the non-radioactive amino acid in separate flasks. Incorporation of the labeled amino acid is determined at 2 h as above. L-arginine: closed line, open circle; L-glutamic acid: open circle; L-valine: closed circle.


260
240
220
200
180
160
140
120
100
80
60
40
20
0
TOTAL CPM (X1000)
20
60
120
180
240
300
400
460
CONCENTRATION (μM) OF AMINO ACID

DISCUSSION

The plasmodium, or assimilative phase of Physarum flavicomum, sclerotizes when exposed to adverse environmental conditions. Sclerotization is a differentiation process wherein growth ceases and a dormant state, the sclerotia, is formed. Paralleling the formation of new cell wall and the other events in this process are the depletion of the cell protein and the catabolism of the amino acids of the cell pool. Amino acid catabolism is not significant in the plasmodia in the presence of a normal supply of nutrients (20). It has been generally accepted that incubation in starvation medium induces sclerotization. However, this work indicates that an imbalance in available nutrients triggers this differentiation. Specifically, the lack of an adequate supply of extracellular amino acids serves to initiate sclerotization.

To fully understand this change in preference for metabolites, this research is concerned with amino acid transport as well as amino acid pool and protein decrease in sclerotizing plasmodia. The first indication of amino acid accumulation by microorganisms was reported in the late 1940's (25). Staphylococcus sp., when cultured in the presence of casein hydrolysate, was found to incorporate large amounts of glutamic acid and lysine. The energy dependence of amino acid transport systems was subsequently determined. Recent reports include isolation of certain macromolecular components of amino acid transport systems (25). Moreover, a vast amount of literature has appeared concerning amino acid transport in a variety of organisms. Specifically, such transport

systems for certain amino acids have been studied in Neurospora crassa, Cryptococcus albidus, Candida utilus, and many other fungi, as well as the bacteria (26-29). In the fungi, amino acid transport in normal growth medium differs from that in starvation conditions. Under normal cultural conditions, fungi display a number of transport systems with narrow specificity. When these cells are incubated in the absence of an adequate food supply, they have an active transport system revealing much broader specificity for substrate (28). The amino acids accumulated intracellularly are found to be incorporated into the soluble amino acid pool and cell protein. Under starvation conditions, the amino acids are observed to be completely catabolized (28).

In various microorganisms, the pools of small molecules and their use as intermediary metabolites have been found to fluctuate with environmental conditions. In the bacteria, comparisons of various macromolecular components, such as ribosomes, nucleic acids, and enzymes, of the vegetative phase and the differentiated spore phase reveal striking similarities (13). The pools of small molecules in vegetative cells, however, are distinctly different from those in the spores. During bacterial sporulation, the amino acid pool is decreased by the release of amino acids to the medium with only glutamic acid found to be retained in any significant amounts (13, 14). The decarboxylation of the endogenous glutamic acid has been shown to be involved in spore germination (30).

Dictyostelium discoideum, a cellular slime mold, also differentiates to a spore state when the food supply is depleted. The biochemical

events associated with this fruiting process have undergone extensive analysis as an example of differentiation (9, 10). As an active transport system for amino acids is lacking in this organism, differentiation is believed initiated by release of the amino acid pool components to the medium (10). Protein and protein metabolites rather than carbohydrates seem to be the major energy source during this differentiation as the rate of glutamic acid oxidation via the tricarboxylic acid (TCA) cycle increases. Carbohydrates stored during vegetative growth are used as precursors of spore cell-wall polysaccharides and for the synthesis of disaccharides stored by the spore (31).

A similar change in metabolic pathways, providing energy for the cell, is observed in Cryptococcus albidus. Under starvation conditions, glutamic acid is actively incorporated in this yeast and used for protein synthesis, as well as being completely catabolized to carbon dioxide (28).

In this study, radioactive assay indicates that P. flavicomum when forming sclerotia also readily incorporates amino acids by way of active transport systems. In fact, valine, arginine, and glutamic acid, as well as components of casein hydrolysate, when present in basal salts medium, appear in the cells within the first hour of incubation. The majority of the amino acids incorporated appear first in the soluble amino acid pool. Protein synthesis is initiated and continues after pool incorporation has ceased and radioactivity is observed decreasing in the pool fraction. The incorporation of radioactivity into protein maximizes, and then the radioactive content decreases without the return of isotope

to the soluble pool or the incubation media. Protein is catabolized and radioactive carbon dioxide is liberated from the cell.

Additionally, the protein and pool, formed by actively growing P. flavicomum plasmodia in the presence of a normal supply of nutrients decreases continuously during sclerotization. There is little release of amino acids or protein to the medium suggesting that amino acids are maintained against a concentration gradient and catabolized as intermediary metabolites.

The basic amino acid, L-lysine, and the antimetabolite, canavanine, are competitive inhibitors for arginine transport in the sclerotizing plasmodia of P. flavicomum as has been reported for growing bacterial cells (25). The lack of inhibition when L-valine is used as a competitor suggests a separate transport system for the basic and neutral amino acids in this slime mold. Similar observations have also been made in bacterial cells, as well as in the fungi (25, 28, 29). In addition to these effects, L-norvaline and L-leucine also inhibit valine accumulation by sclerotizing plasmodia. Aspartic acid is somewhat less effective, but still an inhibitor of glutamic acid transport. A wide range of amino acid concentrations reported effective in Neurospora crassa (29) are also effective in saturating the transport systems of P. flavicomum. High levels of amino acids (valine, arginine, and glutamic acid) saturate the transport system and inhibit the incorporation of more amino acid. Moreover, the inhibition of amino acid incorporation and the cell lysis observed with dinitrophenol included in sclerotizing medium, indicates the necessity for an available energy source during the initial states of differentiation (32).

Of equal interest are protein analyses of sclerotizing cells and the patterns of protein turnover. The cell protein synthesized during the growth phase is steadily degraded when cells are placed in basal salts solution. The components liberated from this protein decomposition are not extensively reused in further protein synthesis but are primarily catabolized. However, the protein synthesized when plasmodia are first exposed to starvation conditions is then apparently formed from the soluble amino acid pool components. The lethal effect observed with cycloheximide, an inhibitor of protein synthesis, in the medium reveals the importance of this initial burst of protein synthesis. Similar findings in D. discoideum suggest that such protein synthesis is responsible for morphogenesis in this cellular slime mold (33). It has been suggested that proteases are intimately linked with differentiation (34). The initial period of protein synthesis occurring in Physarum plasmodia during sclerotization could then be involved in the production of such proteolytic enzymes. On the other hand, a regulatory protein might be synthesized during this time which triggers the activation of proteolytic enzymes already present in the cells.

Such reasoning could provide an explanation for the effects of ammonium ions and casein hydrolysate on protein degradation. These substances have been shown to be inhibitory to the synthesis of proteolytic enzymes (35). A similar role in sclerotizing plasmodia could possibly slow the differentiation process by slowing the depletion of amino acids from the cells. Protein degradation does not occur as readily in the presence of

ammonium ions or casein hydrolysate as under complete starvation conditions. As amino acids are removed from the cells by their catabolism, however, these enzymes may be released from inhibition as cell protein is observed to decrease. When present in basal salts solution, casein hydrolysate is rapidly accumulated by the incubating cells, thereby depleting the supply of amino acids in the medium. After 48 h incubation in salts containing casein hydrolysate, the pool content of the cells has also decreased when compared to plasmodia under normal growth conditions. When the amino acid concentration in the sclerotizing plasmodia, then, reaches a suitably low level, proteolytic enzymes may begin to degrade intracellular protein. Ammonium nitrate, when present in sclerotization media, also affects a decreased rate of protein degradation. Although the amino acid pool components are utilized for energy, protein degradation is depressed and sclerotization delayed when ammonium ion is present at certain concentrations in basal salts medium. The content of ammonium ions in the cell pool is then maintained either by such accumulation by the cells or by the deamination of pool amino acids. Subsequently, the concentration is probably decreased, allowing for the observed protein degradation at 48 h. A multitude of effects have been attributed to both this intracellular ammonium ion concentration and the extracellular ammonium ion concentration in other organisms, including the regulation of L-glutamate uptake, thiourea uptake, and ammonium ion uptake, as well as influencing the activity of glutamate dehydrogenase (36, 37). Similar roles in Physarum, as well as the release of ammonium ions to the cell's

environment, and/or its incorporation into cell wall material, could reduce the concentration present in the cell pool and explain our data. In fact, the pH of the sclerotization medium increases with incubation time when plasmodia are cultured in basal salts and basal salts containing ammonium nitrate or casein hydrolysate (38). Ammonium ions are apparently being returned to these media to some extent. The concentration of ammonium ions present in the cells could also be lowered by combining with extra carbohydrate and being incorporated into cell wall material (a polymer of galactosamine). For instance, when dextrose is present in basal salts solution, the pH of the medium remains constant (38). As reported, there is a greater deposition of cell wall observable in sclerotia with glucose in salts solution than in the absence of glucose. This could account for the stable pH of the medium containing glucose. As ammonium ions are removed from the cell pool by these possible routes, protein degradation could ensue due to relief of the inhibition of the proteolytic enzymes.

Analysis of the soluble amino acid pool of sclerotizing plasmodia offers further information concerning the fate of cellular organic nitrogen reserves during sclerotization. The decreases in amino acid pool and protein of differentiating plasmodia incubated for 48 h and 96 h in basal salts containing casein hydrolysate are intermediate to those of sclerotia in basal salts solution and those of actively growing plasmodia. This reflects the trend of amino acid metabolism observed occurring during sclerotization. To parallel this change in biochemical events, glutamate

dehydrogenase activity has been reported to increase eight-fold during spherulation (4). An NAD-dependent dehydrogenase, this enzyme is reported to be catabolic in nature and as such holds a key position in connecting the metabolism of glutamic acid with the TCA cycle (39). It may normally function to furnish ammonium ions for spherule wall formation.

As the presence of casein hydrolysate and ammonium nitrate in the sclerotization medium retards differentiation, so too does the presence of glutathione. Glutathione, a thiol reducing agent (24), has been reported to stabilize protein synthesizing mechanisms (24), as well as to stabilize cell membranes (40). In its oxidized form in the red blood cell, glutathione also appears to stimulate the hexose monophosphate shunt (41), which is a functional metabolic pathway in sclerotia (8). These effects, or the catabolism of the peptide's amino acids, could explain the inhibiting effects observed with glutathione.

Most intriguing are the results obtained with dextrose included in sclerotization medium Glucose catabolism has been shown to decrease during sclerotization (8). The amino acid pool of plasmodia sclerotizing in the presence of the carbohydrate experiences a substantial decrease and there is a large turnover of cell protein. In fact, the liberation of carbon dioxide from amino acid catabolism in basal salts containing dextrose is similar to that in the salts solution alone. Although uptake is not as rapid initially as in basal salts, amino acids are readily accumulated by cells in salts solution containing dextrose and incorporation into pool and

protein fractions is even greater than in salts medium alone. As an explanation, glucose is used as energy source during the initial stages of sclerotization (8) and amino acids are therefore not the primary catabolite. In comparison, the presence of carbohydrates has been previously reported to delay amino acid accumulation in Cryptococcus albidus due to preferential uptake of carbohydrates instead of amino acids (28). Protein synthesis can thus continue for a while longer in the presence of added quantities of glucose in Physarum. The amino acids could also be contributing to slime production, for greater quantities of this viscous material are produced in the presence of glucose (15).

More stimulatory to differentiation than either ammonium nitrate or casein hydrolysate are those compounds in basal salts influencing sclerotization in a manner similar to basal salts alone or those accelerating the process. While many biochemical roles have been assigned to C-AMP, little is known about its effect in P. flavicomum. Recently, Lynch and Henney (8) have shown the compound to be stimulatory to carbohydrate metabolism in sclerotizing plasmodia. C-AMP has also been shown to affect the membrane permeability in D. discoideum. By influencing the flow of ions into the cell, C-AMP has been proposed to affect the contractile machinery involved in ameboid movement in this cellular slime mold (42). These effects in Physarum plasmodia in basal salts solution could explain the relatively slow streaming in such cells as compared to those cells in salts media alone.

Additionally, diamide stimulates the formation of sclerotia. Diamide, an oxidizing agent of glutathione, is probably serving to eliminate the

peptide's inhibitory effect.

Aminonicotinamide, an analog of nicotinamide which is a precursor of nicotinamide adenine dinucleotide (NAD), may also play a role in controlling glycolysis during spherulation. Its incorporation into NAD produces analogs found to inhibit the activity of certain dehydrogenases (43). Possibly inhibiting glycolysis, this compound could then lead to accelerated amino acid catabolism to provide a supply of energy, rapid depletion of the cell's amino acid pool and protein, and a subsequent faster rate of sclerotization.

While only limited studies concerning the effect of DMSO in micro-organisms have been made, many reports, nevertheless, indicate a reduction of the cellular permeability barriers in plants and animals with this compound (44). When DMSO is present in sclerotization media, it is then possible that the plasmodia are losing amino acids to the medium. In fact, analyses of the cell pool in the presence of DMSO show a drastically low content of amino acids. Correspondingly, there is a sharp decrease in cell protein. This compound might offer interesting possibilities for future research as it has been found to also inhibit amino acid transport in bacteria (45).

The effects of PI, a reported inhibitor of acetylcholinesterase, though interesting, are somewhat more difficult to interpret. Acetylcholinesterase has been reported to be associated with streaming in the Myxomycetes (46, 47). The cell death observed when PI is included in sclerotization medium suggests that this enzyme may play a vital role in Physarum, if, in fact, the compound is inhibiting the enzyme's activity. However, the

lack of information concerning the specific function of acetylcholinesterase in the slime mold makes these morphological observations difficult to explain.

The availability of certain nutrient sources thus seems to play an important role in sclerotization in P. flavicomum. Although the differentiation process is quite complex and the exact regulatory mechanisms are the subject of much controversy, certain trends can be noted. For instance, when plasmodia are transferred to starvation conditions, definite metabolic changes are manifested. There is a rapid turnover and catabolism of cell proteins and pool amino acids. This catabolism of cell organic nitrogen reserves is reproduced even in the presence of glucose, which is metabolized by plasmodia in normal growth conditions. Glucose metabolism is also observed to decrease with sclerotization (8). An initial phase of protein synthesis is apparently necessary for sclerotization to occur. There is ample evidence in the literature concerning the stimulation of certain enzymes during differentiation to suggest that proteases are activated by proteins synthesized during this period or they are themselves synthesized (34). As cell organic nitrogen reserves, amino acids and proteins, are quantitatively removed from the sclerotizing plasmodia, dormancy ensues.

In addition to decreasing the rate of protein synthesis, amino acid deprivation slows the synthesis of stable RNA species and partially inhibits M-RNA synthesis in bacteria (48, 49). Stent and Brenner have proved the existence of a single genetic locus in control of RNA synthesis and refer to

it as the "RC" locus (50). The manner of regulation and function of this "control" gene is not fully understood and interpretations of the effects of amino acid starvation on its activity vary. In the early 1960's, control of RNA synthesis was believed to be mediated by the accumulation of uncharged T-RNA. Upon depleting the amino acid pool of a particular amino acid, the corresponding species of naked T-RNA was thought to complex with and inhibit DNA-dependent RNA polymerase thereby blocking further RNA synthesis (48, 50). In the later 1960's, Morris and DeMoss presented evidence for aminoacyl-T-RNA being the determining factor of RNA polymerase activity (51). Since this report, they have found that polysome degradation and a decrease in RNA synthesis occur simultaneously in cells starved for amino acids (52). Intact protein synthesizing systems, the polysome-aminoacyl-T-RNA complex, thus, seemed to affect continuing RNA synthesis while free ribosomes released during amino acid starvation restricted some step in their own biosynthesis. It has been further postulated that the "RC" locus specifies ribosomal proteins and, while naked R-RNA repressed RNA synthesis, some fraction of the ribosomal proteins acts as an inducer. The reassociation of such a protein with R-RNA thus could release the repressed synthesis of RNA (48).

More recently, guanosine tetraphosphates (ppGpp) have been detected accumulating in bacteria during amino acid starvation (53). If this nucleotide could be converted to a 5'-triphosphate and added to RNA, continued synthesis of the nucleic acid would cease as the 3' OH would be blocked by a phosphate (48). Travers, et al. (54), have also suggested

that the accumulated nucleotide may control RNA synthesis by regulating the association of specific factors with RNA polymerase. These factors have been found to specify the specific RNA chains to be transcribed (54).

Amidst these proposed methods of control of RNA synthesis, exist numerous other theorized mechanisms of regulation. For instance, this decrease in RNA synthesis has also been linked with the presence of specific polyamines, as well as depletion of substrates involved in the enzymatic synthesis of the nucleic acid (48). Therefore, the specific change within the cell, accompanying amino acid starvation, that is, the primary motivator for "RC" control (specifically, decrease in RNA synthesis) remains obscure, and resolution will accompany only the development of new insights and new skills.

Although the source of the most abundant information concerning the metabolic effects of amino acids has been the bacterial system, amino acids have also been found influencing various anabolic activities of eukaryotic cells. For instance, certain concentrations of amino acids have been found stimulating protein and RNA synthesis in rat liver (55, 56). Various explantations have been proposed, including the increase in concentration of charged T-RNA in the cell by amino acid stimulation of aminoacyl-T-RNA synthetases, and/or by enhancing the acceptor ability of T-RNA molecules (55). Others suggest that uncharged T-RNA is the predominating factor as it inhibits protein synthesis at a suitably high level as well as fostering polysome degradation (56). In young chicks, proper quantities of amino acids have also been found to increase RNA

synthesis (57). Moreover, certain amino acids have been found to increase uracil incorporation into RNA in Candida utilus (58). It is suggested that the primary effect here, however, may be amino acid stimulation of the transport system responsible for uracil accumulation by the cells. The synthesis of various cellular enzymes has also been shown to be affected by certain amino acids. In Neurospora, for instance, tryptophan in the cell pool has been shown repressing synthesis of tryptophan synthetase (59). Matchett, et al. (59), have subsequently proposed that amino acids are acting as effector molecules in protein synthesis; inducing or repressing the synthesis of proteins as metabolic conditions vary. As in bacterial cells, then, the specific effect of particular concentrations of amino acids in eukaryotic cells deserves further investigation.

Meanwhile, studies of cell components in the vegetative and differentiated phase of a variety of systems suggest a vital role for low molecular weight compounds in differentiation. The role that amino acids, as well as proteins, may play in cancer biochemistry has been the subject of investigation for several years. According to Holley (60), cells undergoing proliferative growth may experience some permeability change resulting in greater accumulation of such nutrient components within the cell. This increase in concentration of cell metabolites may provide for enhanced growth characteristic of cancer cells. Roberts and Simonsen (61) report amino acid catabolism occurring via glutamate and the TCA cycle in Yoshida tumor cells. In Ehrlich ascites tumor cells, the rate of

ribosomal RNA synthesis also is found to be much higher in cells incubated in a medium enriched with amino acids than in a medium deficient in these metabolites (62). Therefore, further investigation of the role of amino acids, and other such low molecular weight metabolites, in differentiating cells may offer vital information regarding the induction and regulation of cancer cells.

SUMMARY

Differentiation of the plasmodia of Physarum flavicomum variety 1 occurs by the formation of dormant sclerotia when they are transferred to starvation media, or media deficient in amino acids. The rate of this differentiation (sclerotization) process is observed to parallel the rate of decrease of amino acids in the cell pool, as well as the decrease of cell proteins. For instance, extracellular amino acids are inhibitory to sclerotial formation as they effect a concentration of amino acids in the cell pool comparable to that of the vegetative state. Dimethyl sulfoxide in sclerotization media, however, affects a drastic decrease in cell pool amino acid content and cell protein and stimulates differentiation. The ammonia concentration of the plasmodial pool also increases with sclerotization indicating deamination of the amino acids produced by proteolysis. Cellular amino acids are thus found to be extensively catabolized to ammonia and carbon dioxide when plasmodia are incubated in the absence of an adequately balanced supply of nutrients. This catabolism of cellular protein and amino acid reserves is duplicated with glucose present in sclerotization media.

Active transport systems for neutral, basic and acidic amino acids are found functioning in P. flavicomum during sclerotization. When various radioactive amino acids are included in sclerotization medium, the amino acids are readily accumulated by the cells and are incorporated into cell pool and protein fractions. The failure of the neutral amino acid, valine, to inhibit cellular incorporation of arginine indicates that separate transport

systems function for the neutral and basic amino acids analogous to those systems in other microorganisms.

A net decrease in cellular protein is observed during sclerotization. From 0 h to 12 h in basal salts medium, plasmodial cells decrease little in total cell protein, but from 12 h to 48 h, the cells experience a steady decrease in this cell fraction (maximum decrease is 43%). The protein synthesized during active growth of microplasmodia in the presence of an adequate supply of nutrients is steadily degraded in differentiating cells. However, a period of protein synthesis, which is necessary for sclerotization to proceed, occurs during the initial stage of differentiation. The majority of this protein is degraded shortly after its synthesis. The addition of ammonium ions to the salts medium is inhibitory to the overall trend of intracellular protein degradation and the rate of sclerotization is accordingly slowed.

Sclerotization is, therefore, initiated by a decreased spectrum of available exogenous amino acids and occurs readily in the presence of glucose. As does the differentiation process in the cellular slime molds and certain bacterial and fungal systems, sclerotization is characterized by the catabolism of low molecular weight pool components (amino acids) as well as the degradation of cell protein.

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