# BIOCHEMICAL ANALYSES AND NUTRITIONAL EFFECTS ON DIFFERENTIATION (ENCYSTMENT) IN THE MYXOMYCETE, PHYSARUM FLAVICOMUM

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A Thesis

Presented to

The Faculty of the Department of Biology

University of Houston

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Philip Shiu-Lun Chu

May, 1976

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My special thanks to my whole family for financial support during my education in this country.

This thesis is dedicated to Miss Marie Cheng.

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## ABSTRACT

A method of inducing haploid differentiation (encystment) in the myxomycete Physarum flavicomum variety 1 is established. Incubation of mid-log phase myxamoebae-swarm cells from SD medium to basal salts solution at pH 4 gives a high percentage yield of microcysts. The microcysts are viable upon transfer back to growth medium. Intracellular protein and carbohydrate are degraded to a great extent during this process. RNA degradation is observed and condensation of cytoplasmic materials occurs upon encystment. DNA is conserved and less than 10% is degraded after 72 hours incubation.

The cell walls of the mature microcysts are isolated and purified. Chemical analyses on the walls reveal the presence of an amino sugar D-galactosamine and neutral sugars D-glucose, D-galactose and D-ribose. Lipid and protein together are found to constitute more than 40% of the dry weight of the walls. Individual amino acids in the wall protein are identified by amino acid analysis. The microcyst walls also contain 5.8% phosphorus. A total of 91.3% of the components of the purified, dry microcyst walls have been identified.

Addition of the supplements casein hydrolysate (0.5%, w/v), L-leucine, L-isoleucine and L-valine (0.5mg/ml each) to the basal salts delay both the rate of encystment and the degradation of intracellular "growth state" protein. L-Leucine exerts a greater inhibitory effect than either L-isoleucine or L-valine. Further experiments show

that these individual UL-$^{14}$C-amino acids are taken up both during growth and encystment. The addition of glycine, D-glucose and ammonium nitrate to basal salts solution does not affect encystment. Degradation of protein during growth is very small, while a significant amount of protein incorporated during the early phase of encystment is decomposed in the later phase of encystment. However, $^{14}$C-leucine labeled "growth state" proteins are found to be more conserved during encystment and less $^{14}CO_2$ is evolved when compared to $^{14}$C-isoleucine and $^{14}$C-valine labeled "growth state" proteins. This work suggests that a condition of nutrient imbalance, rather than "starvation conditions" initiates the encystment process.

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# INTRODUCTION

The Myxomycete, Physarum flavicomum variety 1 exhibits both haploid and diploid phases during its life cycle (22). The diploid phase is naked multinucleate, pigmented plasmodium and the haploid phase is a naked unicellular, motile myxamoeba-swarm cell. Both phases, upon starvation, form dormant cells with rigid refractile cell walls, namely sclerotia (2N) and microcysts (N). The plasmodium has been grown in synthetic media for years (23), however, it is not until recently that the haploid phase has adapted to grow in the same medium (22).

Encystment is a type of single cell differentiation (6), and such systems are studied extensively as models of differentiation. In the cellular slime molds and soil amoebae, encystment can be induced by the change of tonicity in the medium (15, 40) and cellulose has been found to be the major carbohydrate composing of the microcyst walls (53). Recently, O'Day et al. (42-44) reported a series of hydrolytic and proteolytic enzymes that associate with the encystment-excystment of the cellular slime mold Polyspondylium pallidum. Ultrastructural observation during encystment of the protozoan Acanthamoeba castellanii shows that golgi complex are enlarged and autophagic vacuoles appear. The nucleus releases small buds into the cytoplasm and mitochondrial intracristate granule changes are observed. Also, cytoplasmic con-

densation occurs and the final volume of the cyst is reduced to about 80% of that of the original vegetative cell (4).

Haploid cell differentiation long has been a mystery to the students of Myxomycetes. In the mid 19th century, deBary and Cienkowski (14) studied this process and reported that some of the microcysts formed are not walled. Cadman also found that the microcysts of Didymium iridis are not walled and she suggested that this represents a stage toward disintegration rather than a protective mechanism (14). In 1964, Koevenig (26) described the process of encystment in Physarum gyrosum and Fuligo cinerea in his study of the life cycle of Myxomycetes. Modern encystment studies could only begin in the early 1970's after the establishment of simple synthetic media for haploid cells (12, 21). The encystment process in the Myxomycetes is still virtually unexplored. Encystment has been observed upon the depletion of nutrients in these media. However, such a process is quite undesirable for biochemical analyses. In the later phase of growing in these media, cells are found to lyse and encyst at the same time and therefore it is difficult to control the process. Recently, Turner and Johnson (56) have used the above method for electron microscopic observations on encystment of Physarum polycephalum. Cytoplasmic condensation and nucleus distortion is reported and the authors suggest that this may be the reason that old and mature

microcysts are not viable upon returning to growth media.

In this research, a method for inducing encystment is established and biochemical events such as fate of neutral hexose and protein as well as the turnover of nucleic acids are studied. These experiments are modeled after the recent experiments of Henney and Maxey (23) concerning the induction and metabolism of sclerotia in Physarum flavicomum. Since encystment can be measured by the direct cell count method, the effect of different nutrients on the rate of encystment are determined. The uptake and degradation of some of these supplements are further investigated during this process of differentiation by using radioactive compounds. Another part of this work is the chemical analysis of the microcyst walls. Although analyses of the spore and spherule walls of Physarum polycephalum have been studied (36), as yet the microcyst walls have not been reported. The elucidation of the nature of the encystment process of the genus Physarum will be of great value to our understanding of differentiation processes in general.

# MATERIALS AND METHODS

## Organism and Growth Medium

Pure cultures of myxamoebae-swarm cells of Physarum flavicomum are maintained in semi-defined liquid medium as reported by Henney & Asgari and Henney (21). During each transfer, 200 ml of semi-defined medium are sterilized in 500 ml, cotton-plugged, Erlenmeyer flasks, and 0.2 ml of sterile 0.25% (w/v) hematin solution in 1% (w/v) NaOH is added. From a well-dispersed mid-log phase culture with a cell number of about $10^7$ cells/ml, 5 ml of myxamoebae are transfered to each flask of medium. All the cultures are kept in the dark at 25°C for about 4 days on a gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N. J.) at 150 rpm.

## Encystment Conditions

Encystment is induced by incubation of myxamoebae-swarm cells in a non-nutrient solution of basal salts (BS) (Table 1). Actively growing mid-log phase myxamoebae in semi-defined media are transfered to sterile 250 ml centrifuge bottles (Nalgene). The cells are centrifuged at 5,000 rpm for 10 min at 25°C in the GSA rotor of a Sorvall model RC2-B refrigerated centrifuge (Ivan Sorval, Inc., Norwalk, Conn.). The medium is discarded and the cells are washed

Table 1. The composition of the basal salts solution[a] used in encystment experiments.

| Component | Gram/liter |
|---|---|
| Citric Acid | 2.978 g |
| $K_2HPO_4$ | 3.310 g |
| NaCl | 0.250 g |
| $MgSO_4 \cdot 7H_2O$ | 0.100 g |
| $CaCl_2 \cdot 2H_2O$ | 0.050 g |

[a] pH of the basal salts solution is at 4

twice with 200 ml of sterile basal salts solution and finally resuspended in 200 ml of basal salts solution and incubated on the shaker for a period of 72 hours.

## Direct Cell Count

During encystment, naked myxamoebae-swarm cells convert to microcysts with a definite cell wall. The rate of encystment is measured by direct cell counts of encysting cells using a hemocytometer (Neubauer) and a microscope at a magnification of 430x. One milliliter samples are removed from the cultures at 0, 12, 24, 36, 48, 72 hour intervals. A drop of the well mixed samples, diluted 1/10 with basal salts, is used to fill the counting chamber. All the 25 small squares (red blood cell area) in the chamber are examined for the number of cells and the number of microcysts present. A factor of $10^4$ is multiplied to all counts to obtain the actual total number of cells/ml of diluted samples.

## Biochemical Events During Encystment

### A) Fate of Intracellular Protein

To determine the fate of intracellular protein during encystment, 2 ml samples are collected at 0, 12, 24, 36, 48, 72 hour intervals and

washed twice with basal salts solution by centrifugation at 5,000 rpm for 10 min at 0°C. Five milliliters of cold 10% trichloroacetic acid (TCA) are added to the cells, which are then incubated in an ice bath for 30 min. The precipitates are then collected by centrifugation at 10,000 rpm for 10 min. The TCA precipitates are dissolved in 0.4 N NaOH by incubating the samples in a water bath at 100°C for 10 min. The final volume of each sample is adjusted to 2 ml with 0.4 N NaOH. The protein content of the samples are determined by the method of Lowry, et al. (33) using crystallized bovine serum albumin, Fraction V (Sigma Chemical, St. Louis) to prepare the standard curve.

B) Fate of Neutral Hexose

The total content of neutral hexose during encystment is determined by the procedures of Spiro (49) using anthrone (Matheson, Coleman and Bell). At 0, 6, 12, 24, 48, 72 hour intervals, 0.2 ml samples are collected and washed. Five milliliters of ice cold anthrone reagent containing 72% (v/v) concentrated $H_2SO_4$, 0.05% (w/v) anthrone and 1% (w/v) thiourea are added to the samples and mixed. The tubes are then heated in a boiling water bath for 15 min and cooled for 20 min in running water. The optical density of the samples are determined at 620 nm. A standard curve of $OD_{620}$ versus concentration of galactose is used to determine the carbohy-

drate concentrations of the samples.

C) Extraction of Nucleic Acid (45)

Twenty milliliter samples are removed at 0, 24, 48, 72 hour intervals during encystment. The washed cell pellets are treated with 3 ml of cold 1 N $HClO_4$ (Perchloric acid, PCA) and incubated in an ice bath for 24 hours before centrifugation at 10,000 rpm for 10 min at 0°C. The supernatants are collected and designated as the RNA fraction. The cold PCA treated pellets are then subjected to extraction in 3 ml of 0.5 N PCA at 70°C for 30 min. The supernatants are used for DNA determination.

D) Fate of DNA (7)

The diphenylamine reagent is made by dissolving 1.5 g of ethanol recrystallized diphenylamine in 100 ml of redistilled glacial acetic acid with 1.5 ml of concentrated $H_2SO_4$. Prior to use, 0.5% of 1.6% acetaldehyde is added to the reagent. Equal volumes of reagent and DNA extracts are mixed and incubated at 25°C for 18 hours. The optical density of the tubes are read at 600 nm. A standard curve is prepared in the same way as the unknown samples, using 10 to 100 µg of Salmon sperm DNA (Mann Research Lab., N. Y., N. Y.) dissolved in an equal volume of 5 mM NaOH and 1 N PCA.

E) Fate of RNA (25)

The acidic reagent is made by adding 1 ml of 10% (w/v) $FeCl_3 \cdot 6H_2O$ solution to 200 ml of concentrated HCl. The alcoholic orcinol reagent is a 6% (w/v) solution of purified orcinol (Sigma Chemical) in 95% ethanol. Three milliliters of the acidic reagent is added and mixed with 1.5 ml of each sample together with 0.2 ml of the alcoholic orcinol reagent. The tubes are heated for 20 min in a boiling water bath. After cooling, the mixture is diluted with an equal volume of distilled water and the optical density of the tubes are read at 660 nm. D(-) ribose (Sigma Chemical) is used for the preparation of the standard curve and unknowns are compared.

Preparation of Microcyst Walls

Mature microcysts induced in basal salts solution are collected and washed. The microcysts are resuspended in distilled water with approximately $10^9$ cells/ml and the suspension is then passed through a chilled French press (American Instrument Co., Silver Spring, Md.) three times at a pressure maintained above 6,500 psi. Microscopic observation reveal at least 99.9% breakage. Further passes through the French press is avoided since this results in the formation of very small wall fragments. The microcyst wall fraction is collected by centrifugation at 3,000 rpm at $0^{\circ}C$. The walls

are washed with 20 ml of chilled (4°C) distilled water. However, in the later washes, it is difficult to form pellets even at higher centrifugation speed (17,500 rpm). Thus, 0.45 μ millipore HA membrane filter (Millipore Corp., Bediford, Mass.) is finally used to collect the microcyst walls. The final wash fluid contains no material that absorbs at 235, 260, 280 nm. To achieve this purity, at least 15-20 washes are required. The purity of the wall preparation is then checked by phase contrast and electron microscopic examination.

The purified microcyst walls are then "shell frozen" and lyophilized and stored in a desiccator over $CaSO_4$ (Drierite).

## Hydrolysis of Microcyst Walls

Hydrolysis of microcyst walls is done by putting the samples in vacuo for 6 hours at 100°C in 6 N HCl to recover hexosamines or in 2 N $H_2SO_4$ to recover neutral sugars. For the recovery of individual amino acids, 6 N HCl at 110°C for 24 hours in vacuo is used. The humus in the hydrolysates is removed by centrifugation after the hydrolysis. To remove the charged molecules present in the neutral sugar hydrolysate an ion-exchange column containing a layer of both Dowex 50W-X8, 200-400 mesh ($H^+$ form) and Bio-Rad AG1-X8, 200-400 mesh (formate form) is used. The eluate containing the neutral materials is collected. The amino acid

hydrolysate contains both amino acids and hexosamines. In order to remove the hexosamines, a column containing Bio-Rad AG1-X8, 200-400 mesh ($OH^-$ form) is used. The hydrolysate is first dried and readjusted to about pH 10.5. At that pH the hexosamine is not charged and is not adsorbed by the column. The amino acids which adsorbed to the column are collected by eluting the column with 2 N HCl. All samples are dried by flash evaporation and redissolved in suitable amounts of the appropriate solution for further analysis.

Hexosamine Quantitation and Identification

Thin-layer chromatography is used to identify the hexosamine present in the microcyst walls. Eastman precoated cellulose Chromagram plates (160 $\mu$ thick) is sprayed evenly with 0.1 N barium chloride and dried before the samples are applied. The solvent system used is n-butanol-pyridine-water (6:4:3, v/v/v) and the plate is developed twice, ascendingly, in an S-chamber. The spots are visualized by the Morgan-Elson spray as described by Stahl (50).

The Elson-Morgan test is used to quantify the amount of hexosamine in the cell wall hydrolysate. Reagent I is made by adding 2.5 ml acetylacetone and 50 ml of pH 9.6 buffer prepared by mixing equal volumes of 1 M $NaHCO_3$ and 1 M $Na_2CO_3$. Reagent II is made by dissolving 0.8 g of p-dimethylamino-benzaldehyde in 30 ml of absolute ethanol and 30 ml of concentrated HCl. One ml of the sample is mixed with 2 ml of 1 N NaOH with a few drops

of phenolphthalein. One ml of reagent I is added and the tubes are capped with marbles and heated for 20 min in a boiling water bath. After cooling, 5 ml of absolute ethanol is added and followed by 1 ml of reagent II. The final volume of the mixture is adjusted to 10 ml with absolute ethanol and placed in a 65°C water bath for 10 min to liberate the $CO_2$. The optical density of the tubes are read at 530 nm. D-Galactosamine·HCl is used for the preparation of a standard curve.

The hexosamine is further identified by using the acidic and neutral column of the Phoenix Amino Acid Analyzer which is programmed from 30°C to 50°C at 30 min and the buffers from pH 3.25 to pH 4.25 at 180 min. The hexosamine is identified by comparing the elution profile with that of a standard. A 1 μM/ml solution of D-galactosamine·HCl is used as the standard.

Neutral Sugar Quantitation and Identification

The total amount of neutral sugar in the microcyst wall is determined by the anthrone reaction mentioned previously on hydrolyzed samples and non-hydrolyzed samples of purified walls. Identification of the neutral sugar is done by thin-layer chromatography on Eastman precoated cellulose Chromagram plates (160 μ thick) with ethyl acetate-pyridine-water (2:1:2, v/v/v, upper layer) for one dimensional chromatography. The spots are visualized by spraying with 0.05 M aniline hydrogen phthalate in water-saturated n-butanol accord-

ing to Lamkin et al. (29). To identify and quantify the neutral sugars present, Glucostat and Galactostat (Worthington Biochemical Corporation, Freehold, N. J.) reactions are performed on the hydrolysate (58).

## Protein Quantitation and Amino Acid Identification

To quantify the amount of protein, non-hydrolyzed microcyst walls are dissolved in 0.4 N NaOH. The amount in the sample is determined by the Lowry et al. (33) method described previously. To identify the individual amino acids present in the protein, hydrolyzed samples are put onto the acidic and neutral ion-exchange column and basic ion-exchange column of a Phoenix Amino Acid Analyzer. The columns are regenerated by 0.4 N NaOH and the buffers and temperatures used to operate the columns are listed in Table 2. The amount of each amino acid is determined by comparing the height-times-width with a Beckman amino acid standard mixture.

## Lipid Extraction

Fifty-one milligrams of lyophilized microcyst walls are refluxed at 50°C for 12 hours with 20 ml each of the following solvents : a) 1% concentrated HCl in 95% ethanol-ether (1:1, v/v), b) 95% ethanol-ether (1:1, v/v) and c) chloroform. The solvents after extraction are collected and dried in a tared-preweighed planchet. After the

Table 2. Amino acid analyzer buffers

| Component | Buffer pH | | | |
|---|---|---|---|---|
| | 3.25 ± 0.02 | 4.25 ± 0.02 | 4.26 ± 0.02 | 5.28 ± 0.02 |
| | Column I | | Column II | |
| Sodium Concentration | 0.20 N | 0.20 N | 0.38 N | 0.35 N |
| Citric Acid · $H_2O$ | 840 gm | 840 gm | 532 gm | 491 gm |
| NaOH (97%) | 330 gm | 330 gm | 312 gm | 288 gm |
| Concentrated HCl | 426 ml | 188 ml | 307 ml | 136 ml |
| Octanoic Acid | 4.0 ml | 4.0 ml | 2.0 ml | 2.0 ml |
| Thiodiglycol | 200 ml | 200 ml | - | - |
| Brij-35 Solution | 80 ml | 80 ml | 40 ml | 40 ml |
| Final Volume | 40 liters | 20 liters | 20 liters | 20 liters |

Column I : Acidic and neutral column. pH changes from 3.25 to 4.25 and temperature changes from 30°C to 50°C at 180 min and 30 min respectively after zero time.

Column II : Basic column. pH changes from 4.26 to 5.28 and temperature from 30°C to 50°C at 120 min after zero time.

solvents are completely evaporated, the planchet with the extracted lipid is put into an oven at 80°C to achieve constant weight.

Total Phosphate Determination

The total phosphate content of the purified microcyst wall is determined by the method of Fiske and SubbaRow (11). The reducing agent is made by dissolving 0.2 g of 1-amino-2-naphthol-4-sulfonic acid and 1.2 g each of sodium bisulfite and sodium sulfite in 104 ml of distilled water. One milliliter of 5 N $H_2SO_4$ is added to 5 mg of purified wall in a Corex centrifuge test tube. The suspension is heated over a flame. When the content becomes brown, it is cooled and a few drops of 2 N nitric acid are added and heated until white fumes appear and the solution turns colorless. After cooling, the residue is dissolved and adjusted to one ml with distilled water. One milliliter of 5 N $H_2SO_4$ is again added to the sample followed by 1 ml of 2.5% ammonium molybdate. After mixing, 0.1 ml of reducing agent is added and the final volume is adjusted to 10 ml with distilled water, and incubated at room temperature for 10 min. The optical density of the solution is read at 660 nm. A standard solution of 1 μM/ml phosphorus is made for the standard curve by dissolving 136.1 mg $KH_2PO_4$ in 1 liter of distilled water.

Nutritional Studies

A) Chemical Analysis. Mid-log phase myxamoebae grown in SD medium are collected by centrifugation at 5,000 rpm for 10 min and washed twice in BS solution. Washed myxamoebae are then used to inoculate flasks containing BS, BS with D-glucose (0.5%, w/v), ammonium nitrate (12.4 mM), casein hydrolysate (0.5%, w/v, NZ-case, Sheffield Chem. Co., Union, N.J.), glycine, L-valine, L-isoleucine and leucine (0.5 mg/ml each). The final cell number in all these flasks is $10^7$ cells/ml. Two milliliter samples are taken out from each flask at 0, 12, 24, 36, 48, 72 hour intervals. The samples are used for direct cell counts and protein determinations according to the methods previously described.

B) Radioisotope Experiments. A small inoculum ($5 \times 10^5$ cells/ml) is used to start growing myxamoebae in SD medium (200 ml/flask) with UL-$^{14}$C-protein hydrolysate (0.1 $\mu$Ci/ml). $^{14}CO_2$ evolved during growth is collected by 50% KOH contained in a short test-tube suspended inside the flask in a wire basket. The KOH is changed at 24 hour intervals. After 100 hours, the cells reach the mid-log phase. The radioactive SD medium is separated from the cells by centrifugation at 5,000 rpm for 10 min at 25°C. Cells are then washed twice with 25 ml of BS solution to remove the traces of radioactive medium. Washed $^{14}$C-myxamoebae are then inoculated into flasks containing 25 ml of BS, BS with D-glucose (0.5%, w/v),

ammonium nitrate (12.4 mM), casein hydrolysate (0.5%, w/v), glycine, L-valine, L-isoleucine and L-leucine (0.5 mg/ml each). The final cell number is $10^7$ cells/ml in each flask. Two ml samples from each flask are removed at 0, 12, 24, 36, 48, 72 hour intervals. The $^{14}CO_2$ evolved during differentiation under each condition is collected by 20% KOH in a short test-tube suspended inside the flasks with a wire basket. The KOH solutions are changed at each interval when the samples are removed. A sample of KOH is added to Cocktail A (Table 3) for radioactivity determination.

The 2 ml samples removed at each interval are first centrifuged at 5,000 rpm at 0°C for 10 min and the cells resuspended and washed twice in 2 ml of non-radioactive basal salts solution. The supernatants are combined for radioactivity determination.

The washed cells are incubated in cold 10% TCA for one hour in an ice bath. The TCA precipitates are collected by centrifugation at 10,000 rpm for 10 min at 0°C and are refered to as the acid-insoluble part of the cells. The precipitates are resuspended in 0.5 ml of scintillation grade toluene (Beckman) and 1 ml of 10X Hyamine hydroxide (Packard), and the tubes, capped with marbles, are incubated in a water bath at 55°-60°C for 24 hours. After the pellets are completely dissolved in the mixture, the volume of each sample is adjusted to 2 ml by toluene and radioactivity of the samples are determined in Cocktail A.

The supernatants of the TCA treatment are collected and

assayed in Cocktail A to determine radioactivity of the acid-soluble pool of the cells.

C) Uptake of UL-$^{14}$C-Amino Acids and Fate of Radioactive Protein Formed During Growth in Semi-Defined Medium

Myxamoebae-swarm cells grown in SD medium are washed and transfered to 25 ml of SD media with either UL-$^{14}$C-valine or UL-$^{14}$C-isoleucine or UL-$^{14}$C-leucine each at 0.1 $\mu$Ci/ml. $^{14}CO_2$ evolved from the cells is collected by 20% KOH. The cell number in each flask is $2x10^6$ cells/ml. For the study of uptake, 2 ml samples are removed at 24 and 48 hours incubation for the determination of radioactivity in the TCA precipitates (protein), and the media. For the study of the fate of radioactive protein, cells are allowed to incorporate radioactivity from the media for 20 hours. The radioactive media are then replaced by equal volumes of non-radioactive media. Two ml samples at 20, 24, 36, 48 hour intervals are removed for determination of radioactivity in the TCA precipitates and the media.

D) Fate of Radioactive Protein (Formed in Growth Media) During Encystment in Basal Salts

The remaining radioactive cells from the uptake experiments are washed twice with non-radioactive basal salts solution and re-suspended in basal salts solution to induce encystment. One and a

half ml samples are removed at 0, 24, 48, 72 hour intervals and $^{14}CO_2$ evolved is collected by KOH. Radioactivity of the TCA protein and $^{14}CO_2$ in KOH are determined.

E) Uptake of UL-$^{14}$C-Amino Acids and Fate of Radioactive Protein Formed During Encystment in Basal Salts

Mid-log phase myxamoebae-swarm cells are washed twice in BS and transfered to 25 ml of basal salts containing either UL-$^{14}$C-valine or UL-$^{14}$C-leucine or UL-$^{14}$C-isoleucine (0.1 $\mu$Ci/ml each) and $2 \times 10^{-5}$ M of the respective non-radioactive amino acid. The cell number in each flask is $10^7$ cells/ml. For the study of uptake, 2 ml samples are removed at 12, 24, 48, 72 hour intervals for analyses of the TCA precipitates and radioactivity in the media.

Determination of Radioactivity

For the determination of protein radioactivity, 0.1 ml of each sample is added to 10 ml of Cocktail A (Table 3). For the determination of $^{14}CO_2$ in KOH, 0.01 ml of radioactive KOH is added to 15 ml of Cocktail A. All solutions are kept in capped vials and the CPM of the samples are determined in a Packard Tri-Cab Scintillation Spectrometer 3003 set for $^{14}$C. Corrections to DPM are made by adding a known amount of $^{14}$C-toluene to the samples.

Table 3. Cocktail A used for radioactive assays.

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

[a] Primary flour: 2,5 diphenyloxazole.

[b] Secondary flour: 1,4-bis (2-(5-phenyloxazolyl)-benzene).

as internal standard and the percent efficiency is determined by comparing the actual CPM added to the observed CPM.

## Chemicals

UL-$^{14}$C-Valine, UL-$^{14}$C-isoleucine and UL-$^{14}$C-leucine are from New England Nuclear, Boston, Massachusettes and UL-$^{14}$C-Protein hydrolysate (yeast profile) is from Schwarz/Mann, Orangeburg, New York. All other chemicals and organic solvents are either spectro-grade or analytical grade.

## Glassware

All glassware used in the experiments is cleaned in chromic acid and rinsed in tap water at least 12 times and given a final distilled water rinse.

# RESULTS

## Rate of Encystment

Myxamoebae grown in SD medium (Fig. 1A) can be induced to encyst in a non-nutrient basal salts solution at pH 4. First, the myxamoebae round up (Fig. 1B) and finally microcysts with definite refractile cell walls are formed (Fig. 1C). Encystment begins early in the basal salts. For instance, at 12 hours, about 12% of the total population has been changed to microcysts and at 36 hours, about 90% of the cells are cysts (Fig. 2). In addition, an increase of pH from 4 to 4.7 after 48 hours incubation in the basal salts solution is observed. Viability of the microcysts formed is checked by transferring the microcysts back to the SD medium. After 3 to 6 hours, the myxamoebae-swarm cells emerge through a small pore in the cell wall and leave empty cell walls behind (Fig. 1D).

## Fate of Intracellular Protein During Encystment in Basal Salts

To study the rate of protein degradation during encystment, samples are collected at 0, 12, 24, 36, 48, 72 hour intervals. Protein determination on these samples show that protein decomposition is very steady throughout encystment. A decrease in intracellular protein of about 40% occurs after 72 hours incubation in basal salts (Fig. 3).

Fig. 1. Morphological changes during encystment in basal salts solution. A) Myxamoebae (2430x). B) Encysting myxamoebae (2320x). C) Microcysts (2320x). D) Excysting microcysts and 'ghost cells' (2030x). Myxamoebae are grown in SD medium. Encysting myxamoebae are obtained from BS solution after 12 hours incubation and microcysts obtained after 72 hours. Ten ml of 72 hour microcysts from BS are transfered to SD medium and after 3-6 hours, excysting microcysts and 'ghost cells' are obtained.

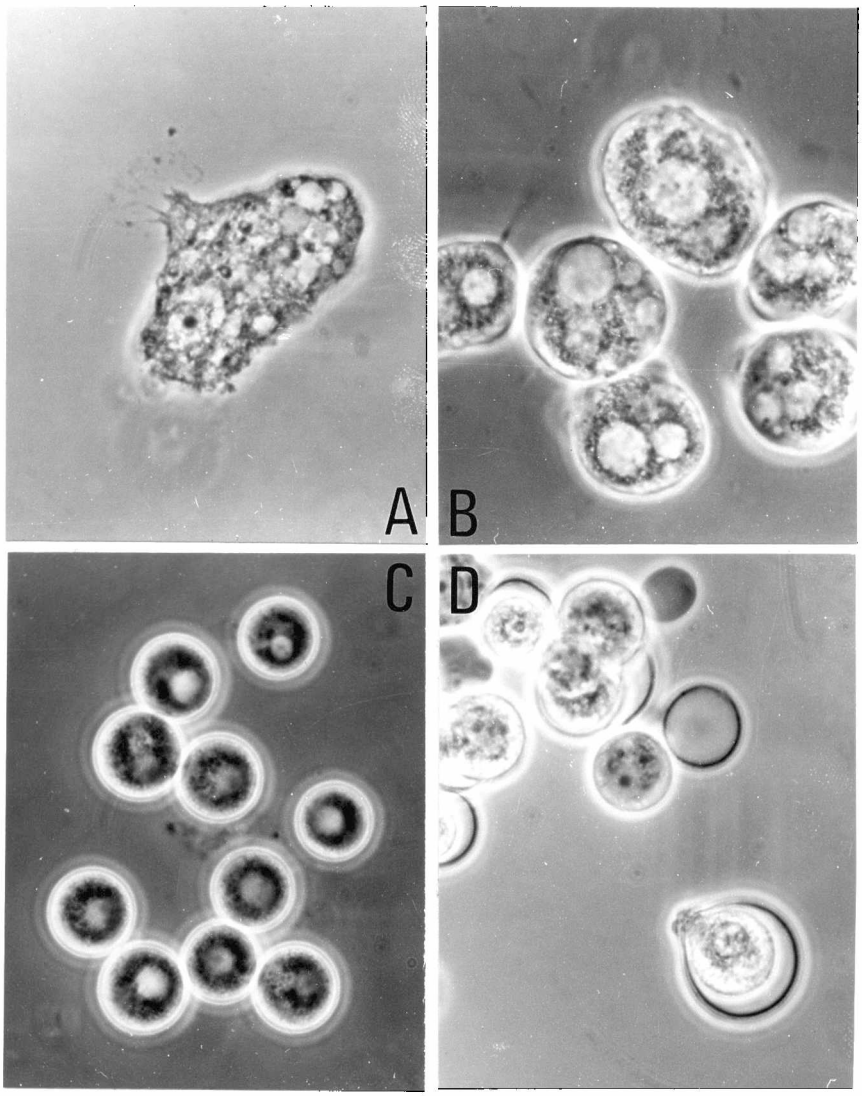
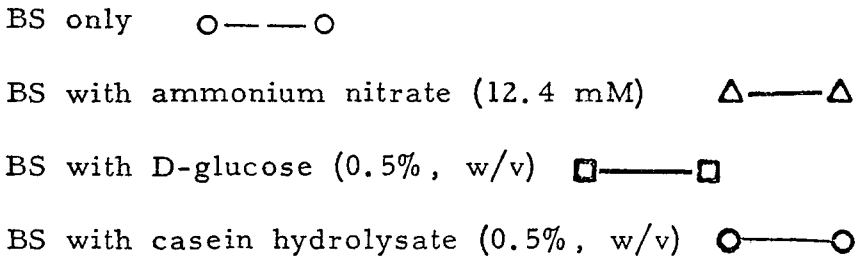
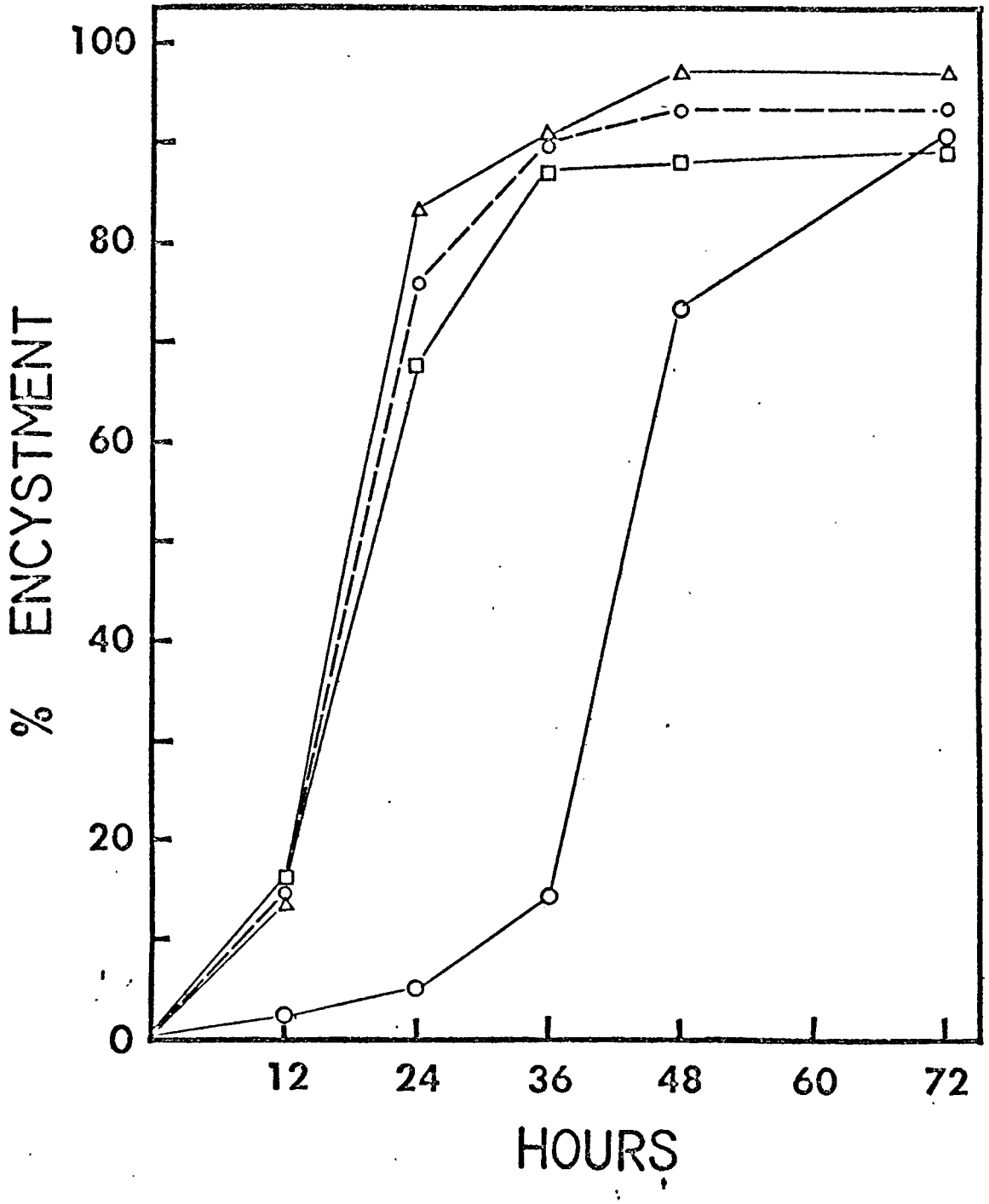

A
B
C
D

Fig. 2. Rate of encystment in basal salts and basal salts with different supplements. Mid-log phase myxamoebae-swarm cells from SD medium are transfered to 200 ml of BS with different supplements and the cell number is adjusted to $10^7$ cells/ml in each experiment. One ml samples are removed at 12 hour intervals and diluted 1/10 with BS for direct cell counts. The percent encystment is expressed as the number of microcysts present in the total population.




100
80
60
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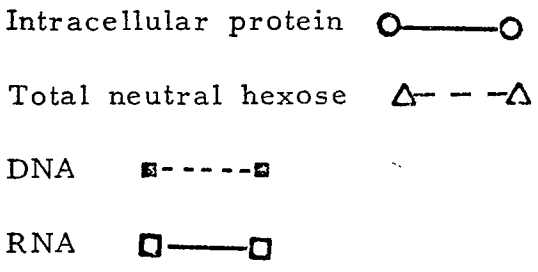
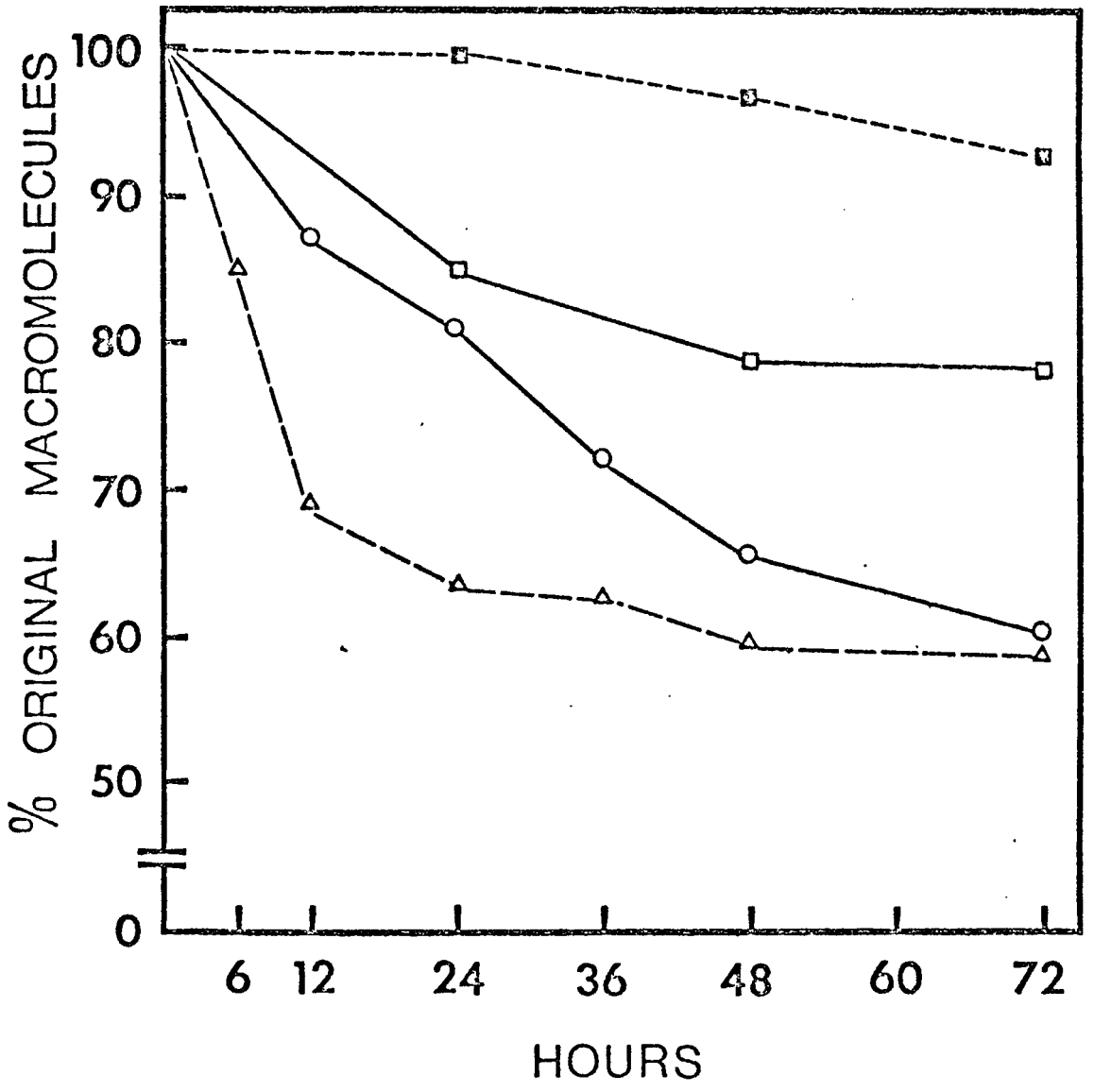
20
0
% ENCYSTMENT
12
24
36
48
60
72
HOURS

Fig. 3. Fate of intracellular macromolecules during encystment in basal salts solution. Mid-log phase myxamoebae-swarm cells are transfered to 200 ml of BS and the cell number is adjusted to $10^7$ cells/ml in each experiment. Two ml and 0.2 ml samples are collected at 12 hour intervals for the determination of intracellular protein and total hexose respectively. Twenty ml of samples are collected 24 hour intervals for the extraction of DNA and RNA.




% ORIGINAL MACROMOLECULES
100
90
80
70
60
50
0
6
12
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36
48
60
72
HOURS

## Fate of Total Neutral Hexose During Encystment in Basal Salts

The content of neutral hexose decreases very rapidly in the encysting myxamoebae. After 12 hours in basal salts a decrease of 31% is observed and a total of 41% is observed by 48 hours (Fig. 3).

## Fate of DNA During Encystment in Basal Salts

The total DNA content changes negligibly during encystment. There is no degradation in the first 24 hours and at 72 hours, less than 10% of the original DNA is degraded (Fig. 3).

## Fate of RNA During Encystment in Basal Salts

Most of the RNA degradation occurs early during encystment. For instance, the decrease in RNA content at 24 hours and 48 hours is 15% and 21% respectively. Further incubation in BS (72 hours) does not result in further RNA degradation (Fig. 3).

## Composition of Purified Microcyst Walls

The appearance of purified microcyst wall fragments, as revealed by both the phase contrast microscope and electron microscope, is illustrated in Fig. 4. The sample does not show any

Fig. 4. Phase contrast and electron microscopic observations of the purified microcyst walls. Mature microcysts from BS are collected and washed with chilled distilled water. The microcysts are resuspended in distilled water with approximately $10^9$ cells/ml and the suspension is then passed through a chilled French press three times at a pressure above 6,500 psi. The microcyst wall fractions are collected by centrifugation at 3,000 rpm at $0^{\circ}C$. Purified walls are obtained after 15-20 washes with cold distilled water.

A) Phase contrast microscopy (2270x)

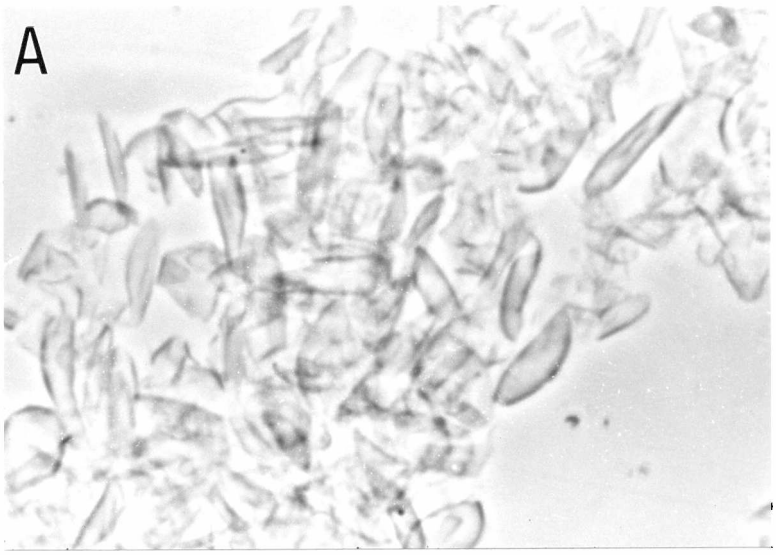
B) Electron microscopy (7333x). The sample for electron microscopy is fixed with 2.5% glutaraldehyde for 3 hours and washed 3 times with 0.15 M sodium cacodylate buffer at pH 7.4. The sample is applied onto the grids with formvar and the excess liquid is absorbed. Then a drop of 1% phosphotungstic acid is put on the grid and after 20 min the excess liquid is removed. Electron micrographs are taken using a Hitachi HS-8 transmission electron microscope.

visible cytoplasmic contamination and the optical density (235, 260, 280 nm) of the supernatant from the final water wash is negligible.

The hydrolysate of hexosamine gives a positive Elson-Morgan reaction. Thin-layer chromatography of the hydrolysate shows a single spot with the same $R_f$ value as D-galactosamine · HCl (Fig. 5). The hydrolysate also shows a single fraction which has the same retention time (230 min) as D-galactosamine · HCl in the acidic and neutral column of the Phoenix Amino Acid Analyzer. The amount of D-galactosamine · HCl present in the hydrolysate as determined in the Elson-Morgan reaction is 32.1% of the dry weight of the microcyst walls.

The microcyst walls also give a positive reaction in the anthrone test. Thin-layer chromatography of the neutral sugar hydrolysate shows the presence of galactose, glucose and ribose (Fig. 6). with glucose as major component and traces of the others. Glucostat and Galactostat reactions on the neutral sugar hydrolysate indicate a composition of 76% glucose and 16% galactose. The remaining 8% may be accounted for by the trace of ribose present. Total neutral sugar determination, using glucose as standard, indicates it accounts for about 10% of the total dry weight of microcyst walls.

The purified dry microcyst walls are found to contain about 20% protein by the method of Lowry et al. (33). Amino acid analyses on the hydrolysates reveal the presence of 14 amino acids from


A

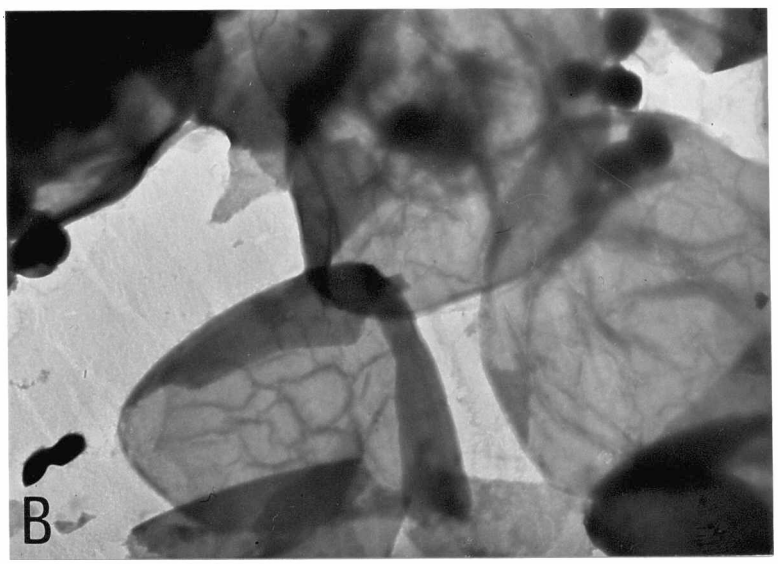

B

Fig. 5. Thin-layer chromatography-amino sugars (one-dimensional)

| Spot No. | Amino sugars | Amount spotted (μg) | $R_f$ | $R_g$ |
|---|---|---|---|---|
| 1 | D-Mannosamine·HCl | 10 | 0.528 | 1.19 |
| 2 | D-Glucosamine·HCl | 10 | 0.504 | 1.13 |
| 3 | D-Galactosamine·HCl | 10 | 0.445 | 1.00 |
| 4 | Cell wall hydrolysate | 15 | 0.438 | 0.98 |
| 5 | N-Acetyl-Galactosamine | 10 | 0.508 | 1.14 |
| 6 | N-Acetyl-Glucosamine | 10 | 0.554 | 1.24 |
| 7 | N-Acetyl-Mannosamine | 10 | 0.608 | 1.37 |

Plate : Eastman precoated cellulose TLC (160μ thick) treated with 0.1 M $BaCl_2$.

Solvent system : n-butanol : pyridine : water (6:4:3, v/v/v). This plate was developed twice in the same dimension.

Spray system : Morgan-Elson spray according to Stahl (50).

$R_f$ : Distance moved by sample/Distance moved by solvent.

$R_g$ : $R_f$ of samples/$R_f$ of D-galactosamine·HCl.

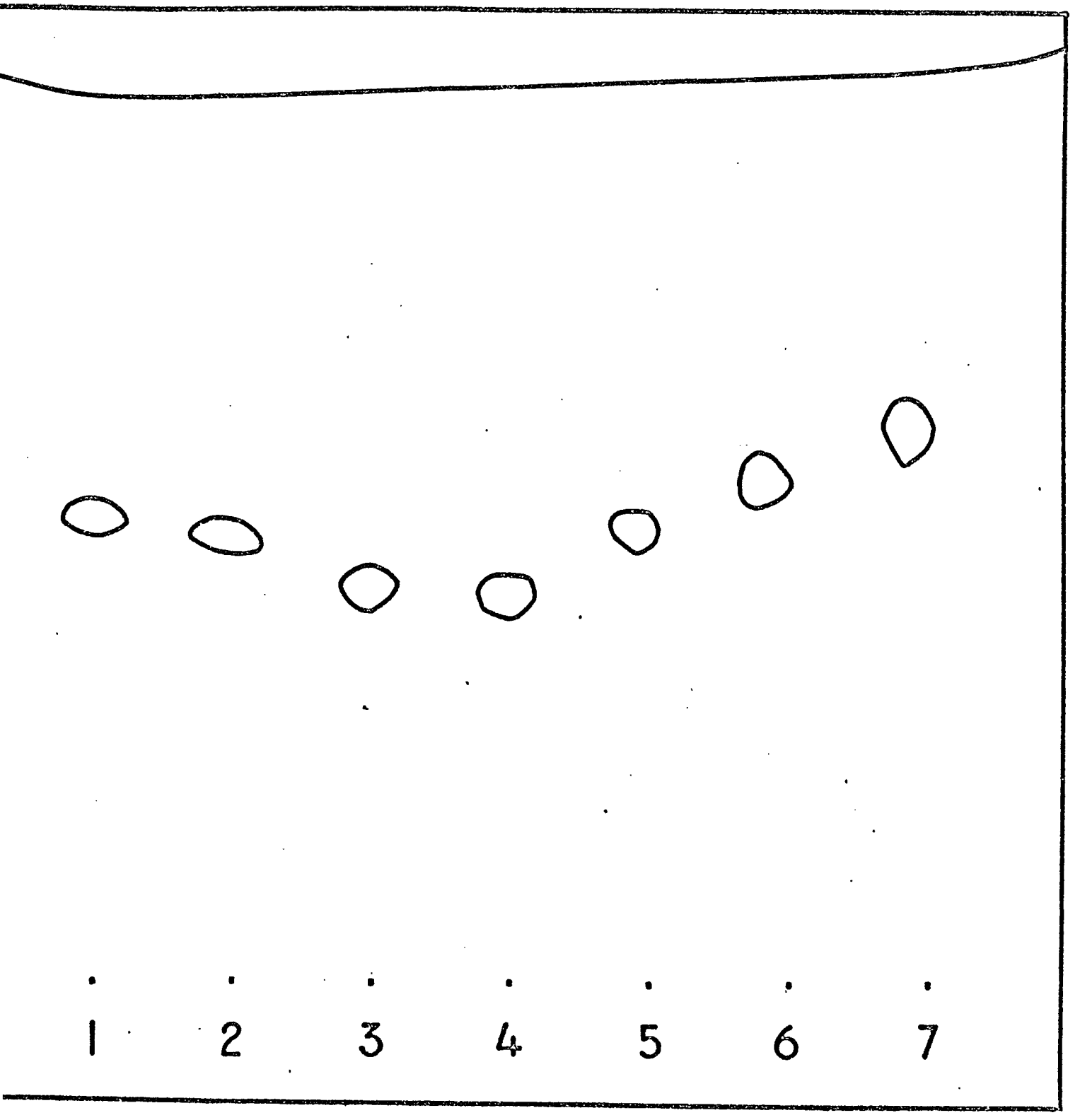

1
2
3
4
5
6
7

Fig. 6. Thin-layer chromatography-neutral sugars (one-dimensional)

| Spot No. | Neutral Sugars | Amount Spotted (µg) | $R_f$ | $R_g$ |
|---|---|---|---|---|
| 1. | D-Mannose | 10 | 0.504 | 1.13 |
| 2 | D-Glucose | 10 | 0.446 | 1.00 |
| 3 | D-Galactose | 10 | 0.403 | 0.90 |
| 4 | Microcyst Unknown | 10 | a)0.401 | 0.90 |
| | | | b)0.442 | 1.00 |
| | | | c)0.636 | 1.43 |
| 5 | D-Mannose | 4 | 0.406 | 0.91 |
| | D-Glucose | 4 | 0.445 | 1.00 |
| | D-Ribose | 2 | 0.641 | 1.44 |
| 6 | D-Ribose | 10 | 0.643 | 1.44 |
| 7 | D-Fucose | 10 | 0.586 | 1.31 |

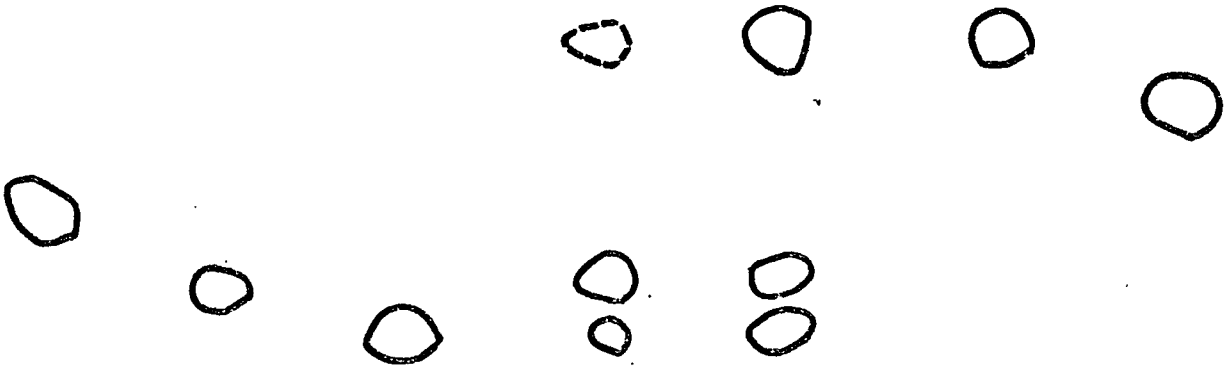
Plate : Eastman precoated cellulose TLC plate (160µ thick).

Solvent system : Ethyl acetate : pyridine : water (2:1:2, v/v/v, upper phase). This plate was developed twice in the same dimension.

Spray system : 0.05 M Aniline hydrogen phthalate in water-saturated n-butanol (29).

$R_f$ : Distance moved by sample/Distance moved by solvent.

$R_g$ : $R_f$ of samples/$R_f$ of D-Glucose.



1 2 3 4 5 6 7

the acidic and neutral column (with traces of half-cystine (as cysteic acid), methionine) and 3 amino acids from the basic column (small amounts of histidine and arginine). The amounts of the individual amino acids as mole percent are shown in Table 4.

Total phosphate content of 5.8% of the dry weight of the wall is determined by the method of Fiske and SubbaRow (11).

Extractions reveal that a total of about 21% lipid is present in the dried microcyst walls.

## Nutritional Effects on Encystment

### A) Direct Cell Counts

D-Glucose (0.5%, w/v), ammonium nitrate (12.4 mM) and glycine (0.5 mg/ml) do not affect the rate of encystment (Fig. 2). However, casein hydrolysate (0.5%, w/v) produces a pronounced effect, and at this concentration, myxamoebae increase in numbers in the BS from $10^7$ cells/ml to $1.7 \times 10^7$ cells/ml in the first 36 hours of incubation with only about 15% of the total population forming cysts. After this period, the cells starts to encyst at a tremendous rate and at 72 hours, about the same percent encystment as the control is reached (Fig. 2). L-Leucine, L-isoleucine and L-valine (0.5 mg/ml) all delay the rate of encystment. After 12 hours incubation, less than 5% of the cells in these media

Table 4. Mole % of amino acids from purified microcyst walls.

| Amino acids | Mole % [a] |
|---|---|
| Aspartic Acid | 10.1 ± 1.0 |
| Threonine | 6.1 ± 1.6 |
| Serine | 5.8 ± 0.8 |
| Glutamic acid | 8.1 ± 1.2 |
| Proline | 6.3 ± 0.1 |
| Glycine | 8.4 ± 0.0 |
| Alanine | 9.5 ± 0.8 |
| Half cystine[b] | 1.0 ± --- |
| Valine | 6.7 ± 0.3 |
| Methionine | Trace |
| Isoleucine | 4.8 ± 0.2 |
| Leucine | 5.7 ± 0.2 |
| Tyrosine | 2.9 ± 0.0 |
| Phenylalanine | 3.8 ± 0.1 |
| Lysine | 16.4 ± 0.5 |
| Histidine | 2.8 ± 0.1 |
| Arginine | 2.4 ± 0.9 |

[a] Mean of two different preparations ± average deviation from mean.

[b] Determine as cysteic acid (37).

Table 5. Chemical composition of microcyst walls.

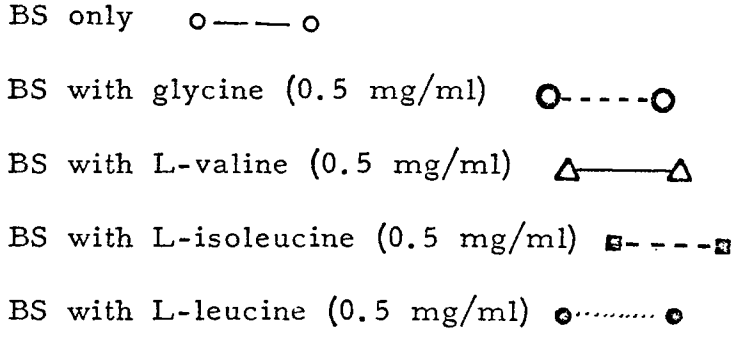
| Components | Percentage |
|---|---|
| Hexosamine (D-galactosamine·HCl) | 32.1 |
| Neutral sugars | 10.4 |
| Total inorganic phosphorus | 5.8 |
| Total lipid | 21.8 |
| Total protein | 21.2 |
| Total percent identified | 91.3 |

encyst. At 72 hours, the percent microcyst yields in L-valine, L-isoleucine and L-leucine are 85%, 79.8% and 71.5%, respectively, as compared to the basal salts control which has a final yield of 93.3% (Fig. 7). Since casein hydrolysate contains 18 different amino acids, other amino acids besides L-valine, L-isoleucine, L-leucine and glycine are also tested for their effect on encystment. The results indicate that there is no marked delay on the rate of encystment in the presence of the amino acids L-aspartic acid, L-serine, L-threonine, L-glutamic acid, L-proline, L-alanine, L-cystine, L-methionine, L-tyrosine, L-phenylalanine, L-lysine, L-histidine and L-arginine (0.5 mg/ml).

B) Fate of Protein During Encystment of Myxamoebae Labeled with $^{14}C$-Protein Hydrolysate

$^{14}C$-Myxamoebae, grown in UL-$^{14}C$-protein hydrolysate, are incubated in BS (control), and BS with different supplements. Samples are collected and the acid-insoluble TCA precipitates are analysed for radioactivity. The fate of protein in BS supplemented with D-glucose and ammonium nitrate are almost the same as the control, while the glycine supplemented BS shows the same pattern for the first 36 hours but protein degradation is greater than the control at 72 hours (Fig. 8). On the other hand, the group of supplements that delay the rate of encystment also delay the rate

Fig. 7. Rate of encystment in basal salts and basal salts with different amino acids. Method for obtaining the percent encystment is the same as in Fig. 6.



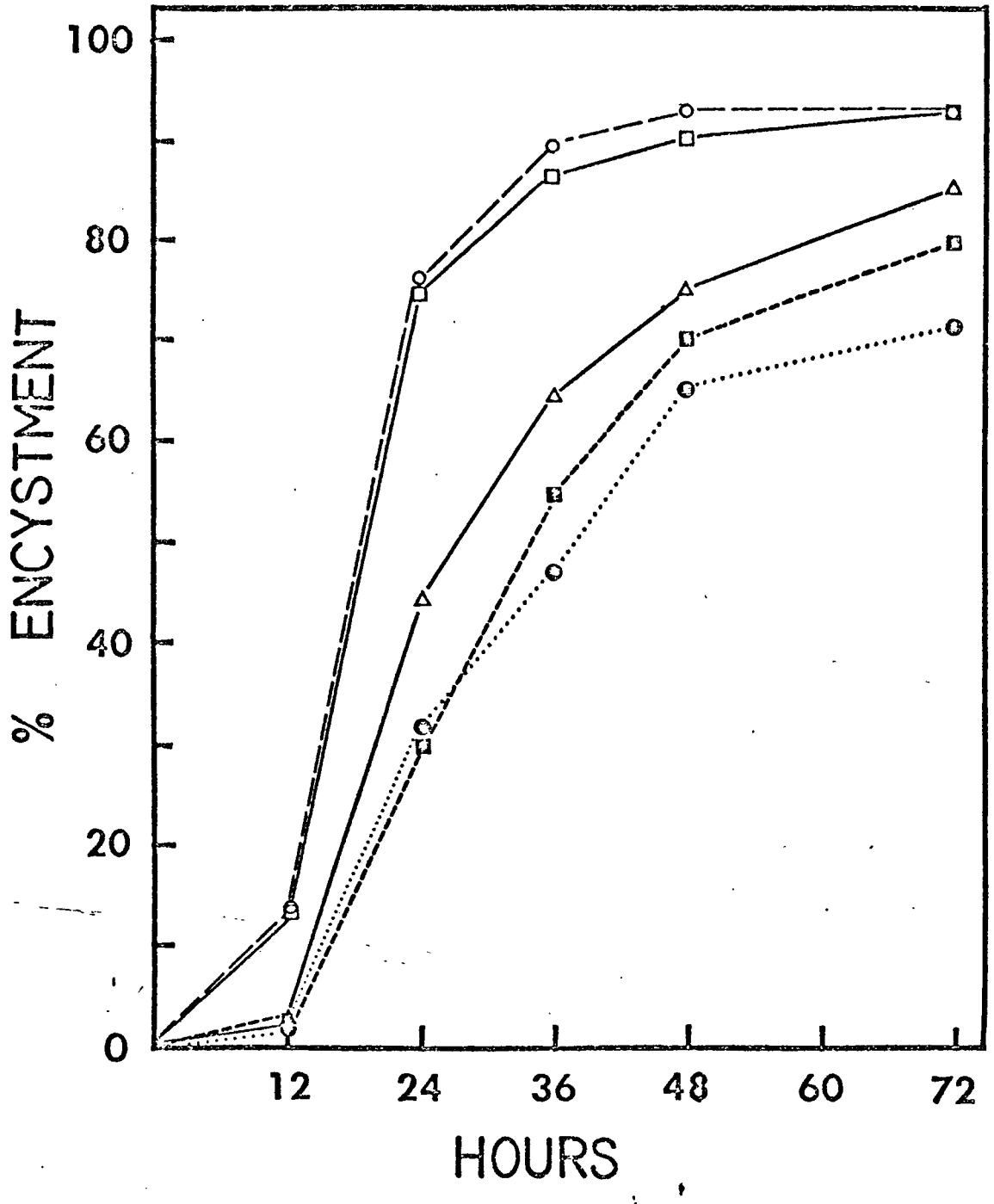

100
80
60
40
20
0
% ENCYSTMENT
12
24
36
48
60
72
HOURS

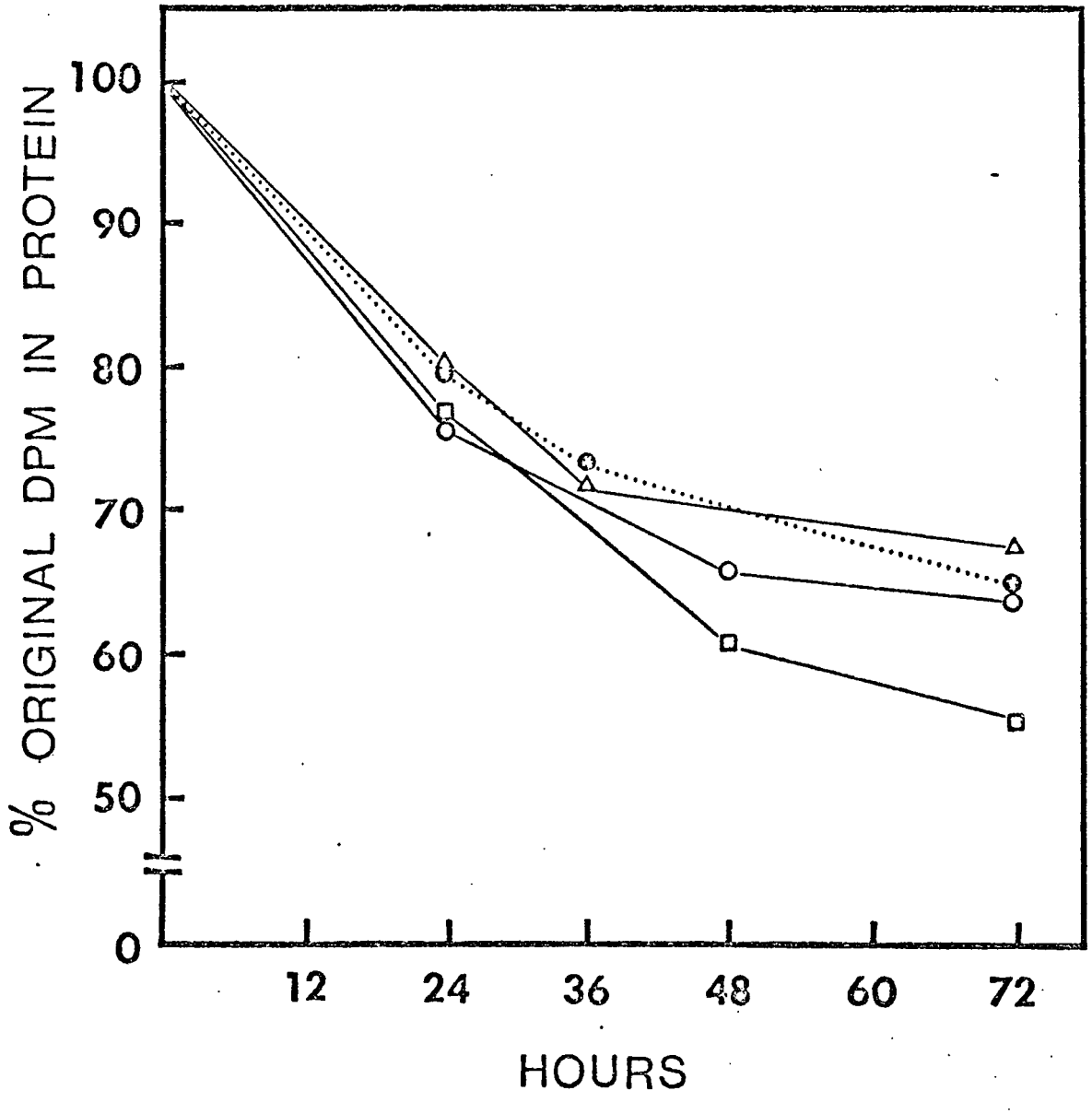
Fig. 8. Fate of DPM in protein during encystment of $^{14}C$-myxamoebae-swarm cells in basal salts with different supplements. Myxamoebae-swarm cells are grown in SD medium conatining 0.1 μCi/ml of UL-$^{14}C$-protein hydrolysate for 48 hours. The mid-log phase $^{14}C$-cells are transfered to 25 ml of non-radioactive BS with different supplements and the cell number is adjusted to $10^7$ cells/ml in each experiment. Two ml samples are collected for analysis of DPM in TCA precipitates.

BS only ○——○

BS with ammonium nitrate (12.4 mM) △——△

BS with D-glucose (0.5%, w/v) ●.....○

BS with glycine (0.5 mg/ml) □——□

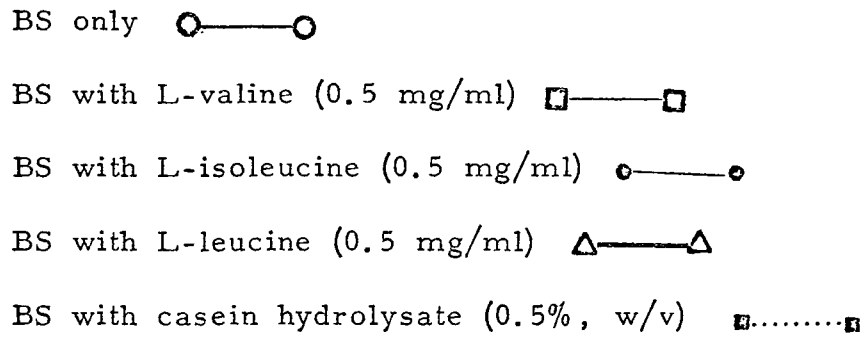

% ORIGINAL DPM IN PROTEIN
100
90
80
70
60
50
0
12
24
36
48
60
72
HOURS

of protein degradation (Fig. 9). L-Leucine produces the greatest effect with only 1.8% degradation in the first 24 hours and considerably less degradation than BS control at 48 and 72 hours (63.9%). Casein hydrolysate, L-isoleucine and L-valine also show effect but to a lesser extent (Fig. 9). The delay in protein degradation produced by casein hydrolysate is overcome between 48-72 hours and the total protein degraded at 72 hours (55.8%) is greater than the control (Fig. 9).

C) Uptake and Fate of UL-$^{14}$C-Amino Acids During Growth and Encystment

Since L-valine, L-isoleucine and L-leucine are found to delay encystment, the rate of uptake of these three amino acids during growth and encystment is determined. Table 6 shows the total DPM in the TCA precipitates after 48 hours incubation as the percent of DPM administered to the cells grown in different UL-$^{14}$C-amino acids. UL-$^{14}$C-Leucine is taken up into protein more than UL-$^{14}$C-isoleucine and UL-$^{14}$C-valine, respectively. However, $^{14}CO_2$ evolved during growth on UL-$^{14}$C-leucine is small. This is further confirmed by incubating the labeled cells (grown for 20 hours in different UL-$^{14}$C-amino acids) in non-radioactive SD medium. The amount of protein degraded is very small and, in each case, after 28 hours incubation in the non-radioactive medium 8% or less

Fig. 9. Fate of DPM in protein during encystment of $^{14}C$-myxamoebae-swarm cells in basal salts with different supplements. Method for obtaining DPM in TCA precipitates is the same as in Fig. 8.



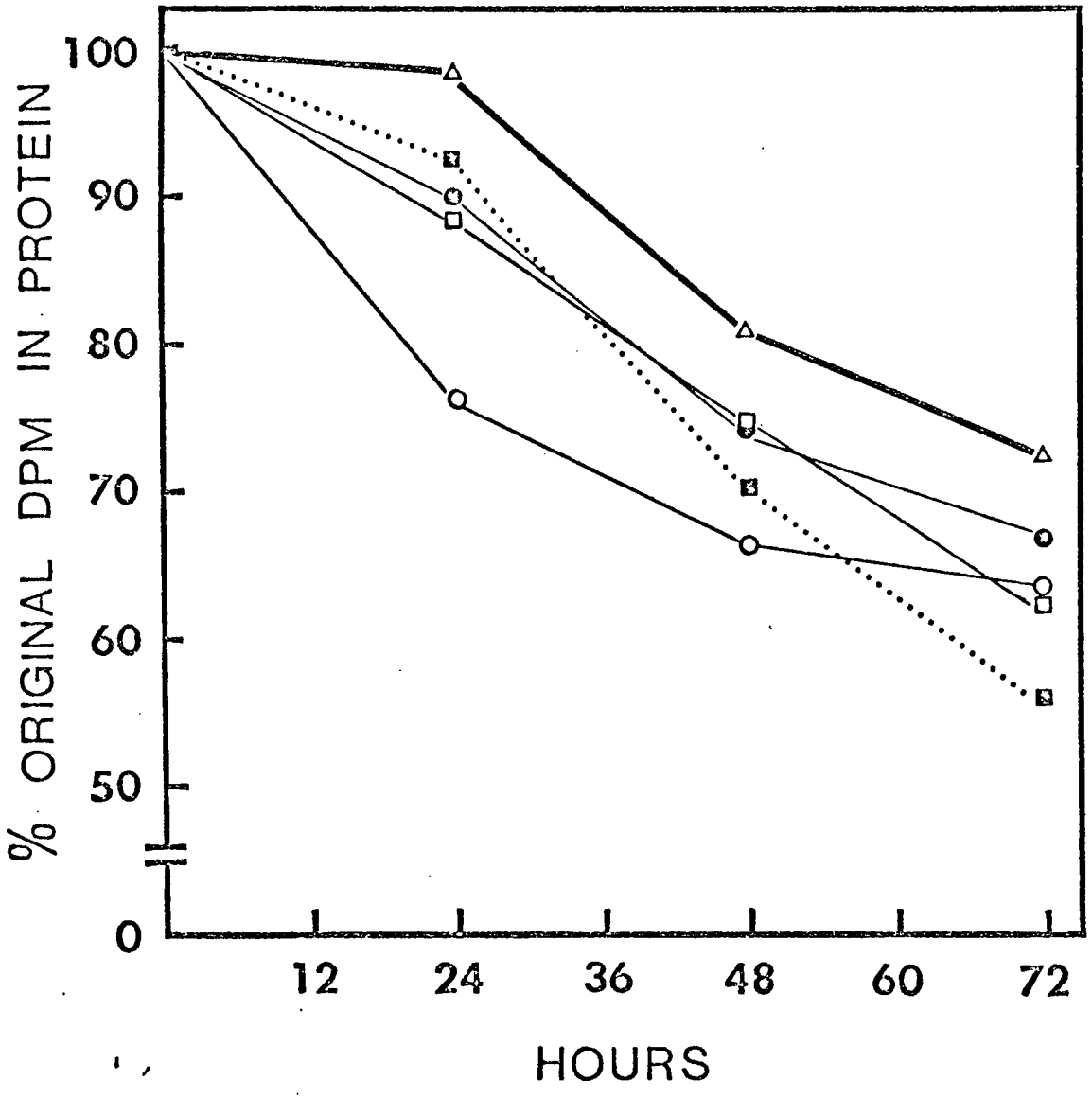

% ORIGINAL DPM IN PROTEIN
100
90
80
70
60
50
0
12
24
36
48
60
72
HOURS

Table 6. Uptake and $^{14}CO_2$ evolved from UL-$^{14}$C-amino acids during growth of myxamoebae in SD medium.

| Radioactive Amino Acids | % Administered DPM[a] in protein[b] | % Administered DPM[a] as $^{14}CO_2$[c] |
|---|---|---|
| UL-$^{14}$C-Valine | 14.2% | 2.6% |
| UL-$^{14}$C-Isoleucine | 16.6% | 1.6% |
| UL-$^{14}$C-Leucine | 18.2% | 0.9% |

[a] A total of $6 \times 10^6$ DPM of each amino acid in 25 ml SD medium.

[b] Total DPM in 48 hour TCA precipitates.

[c] Cumulative $^{14}CO_2$ evolved after 48 hour incubation.

of the labeled proteins are degraded (Table 7).

During encystment, the rate of UL-$^{14}$C-amino acid uptake is very fast in the first 12 hours and then slows down (Fig. 10). At 48 hours, maximum incorporation is achieved and further incubation (72 hours) shows a slight decrease of DPM in TCA precipitates (Fig. 10). The encysting myxamoebae take up UL-$^{14}$C-leucine faster than UL-$^{14}$C-valine and UL-$^{14}$C-isoleucine, respectively. The percent of total DPM in the TCA precipitates in the encysting myxamoebae after 48 hours incubation, when compare to the original radioactivity administered present in the BS, is 20.7%, 18.5% and 23.3% respectively in UL-$^{14}$C-valine, UL-$^{14}$C-isoleucine and UL-$^{14}$C-leucine after 72 hours incubation. $^{14}CO_2$ evolution from the encysting myxamoebae on UL-$^{14}$C-leucine is also found to be less than the other two UL-$^{14}$C-amino acids tested (Fig. 11).

Also myxamoebae-swarm cells are labeled with different UL-$^{14}$C-amino acids for 10 hours in BS and then transfer to non-radioactive BS for the rest of incubation. The decrease in DPM in TCA precipitates is distinct after an additional 14 hours of incubation (Fig. 12). Finally, at the 62 hours incubation period in the non-radioactive medium, 70.5%, 73.5% and 80.2% of the labeled protein remains in the cells labeled with UL-$^{14}$C-valine, UL-$^{14}$C-leucine and UL-$^{14}$C-isoleucine, respectively (Fig. 12).

Table 7. Fate of intracellular $^{14}C$-protein during growth in SD medium.

| Radioactive amino acids | % protein DPM remaining[a] |
|---|---|
| UL-$^{14}C$-valine | 92.0 |
| UL-$^{14}C$-isoleucine | 95.5 |
| UL-$^{14}C$-leucine | 97.2 |

[a] After 28 hours incubation in non-radioactive media.

Fig. 10. Uptake of UL-$^{14}$C-amino acids in TCA precipitates during encystment in radioactive basal salts media. Mid-log phase myxamoebae-swarm cells grown in SD medium are transfered to 25 ml of BS with different UL-$^{14}$C-amino acids and the cell number is adjusted to $10^7$ cells/ml. The flasks also contain a $2 \times 10^{-5}$ M concentration of the same non-radioactive amino acids. Two ml samples are collected at 0, 12, 24, 48, 72 hour intervals for analyses of DPM in TCA precipitates.

UL-$^{14}$C-valine (0.1 μCi/ml) O ——— O

UL-$^{14}$C-isoleucine (0.1 μCi/ml) △ ——— △

UL-$^{14}$C-leucine (0.1 μCi/ml) □ ——— □

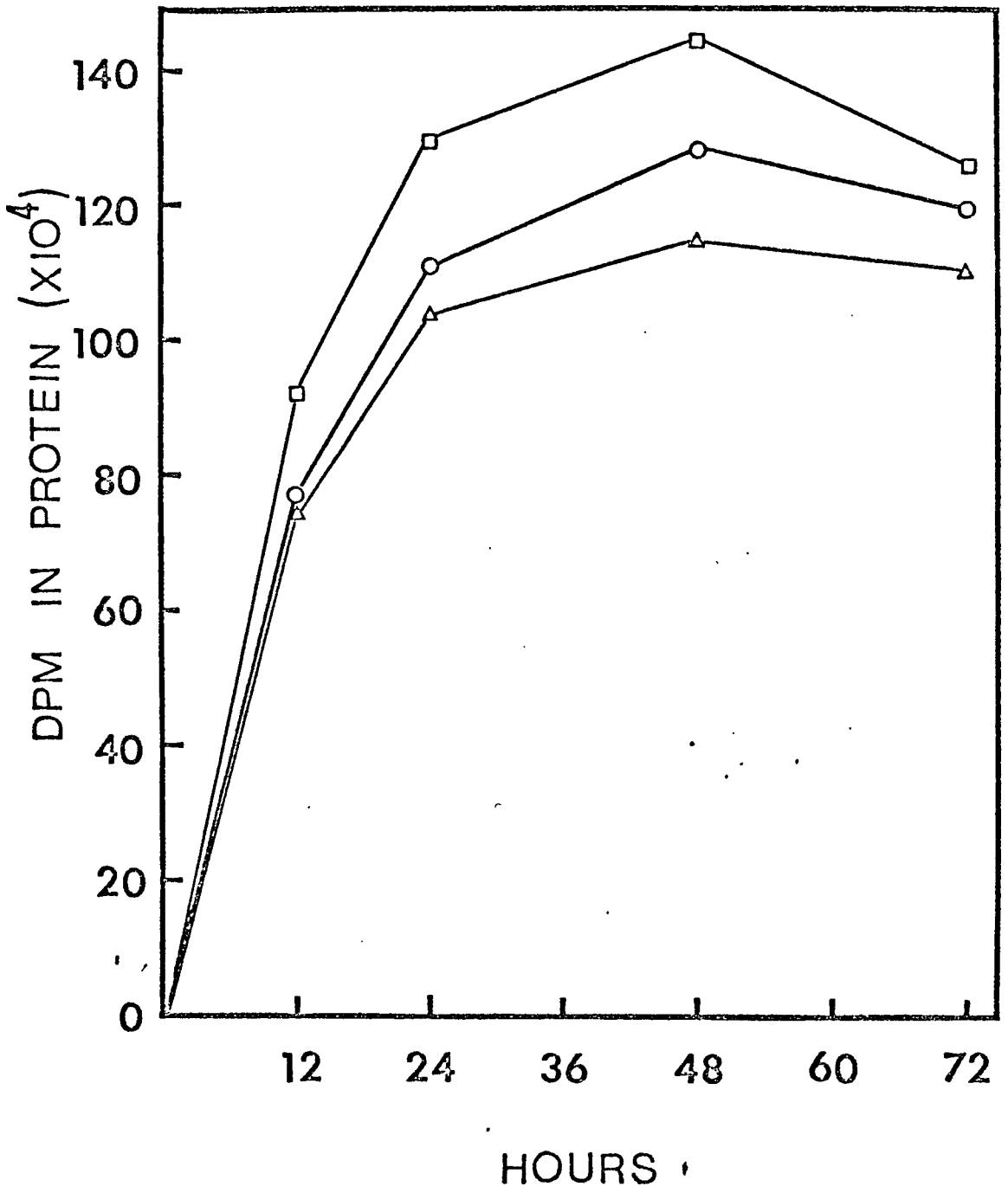

DPM IN PROTEIN (X10⁴)
140
120
100
80
60
40
20
0
12
24
36
48
60
72
HOURS

Fig. 11. Cumulative and interval $^{14}CO_2$ evolved during encystment in basal salts with different UL-$^{14}$C-amino acids. Mid-log phase myxamoebae-swarm cells grown in SD medium are transfered to 25 ml of BS with different UL-$^{14}$C-amino acids and the cell number is adjusted to $10^7$ cells/ml in each experiment. The same non-radioactive amino acids, at a concentration of $2 \times 10^{-5}$ M, are also added to the respective flask. The $^{14}CO_2$ evolved is collected by the 1 ml 20% KOH in the small test tube hanging inside the flask with a wire basket. KOH is changed at 12, 24, 48, 72 hour intervals. Radioactivity in the KOH is determined and both the cumulative and interval $^{14}CO_2$ evolved are plotted in each case.

UL-$^{14}$C-valine (0.1 μCi/ml) O——O

UL-$^{14}$C-isoleucine (0.1 μCi/ml) △——△

UL-$^{14}$C-leucine (0.1 μCi/ml) □——□

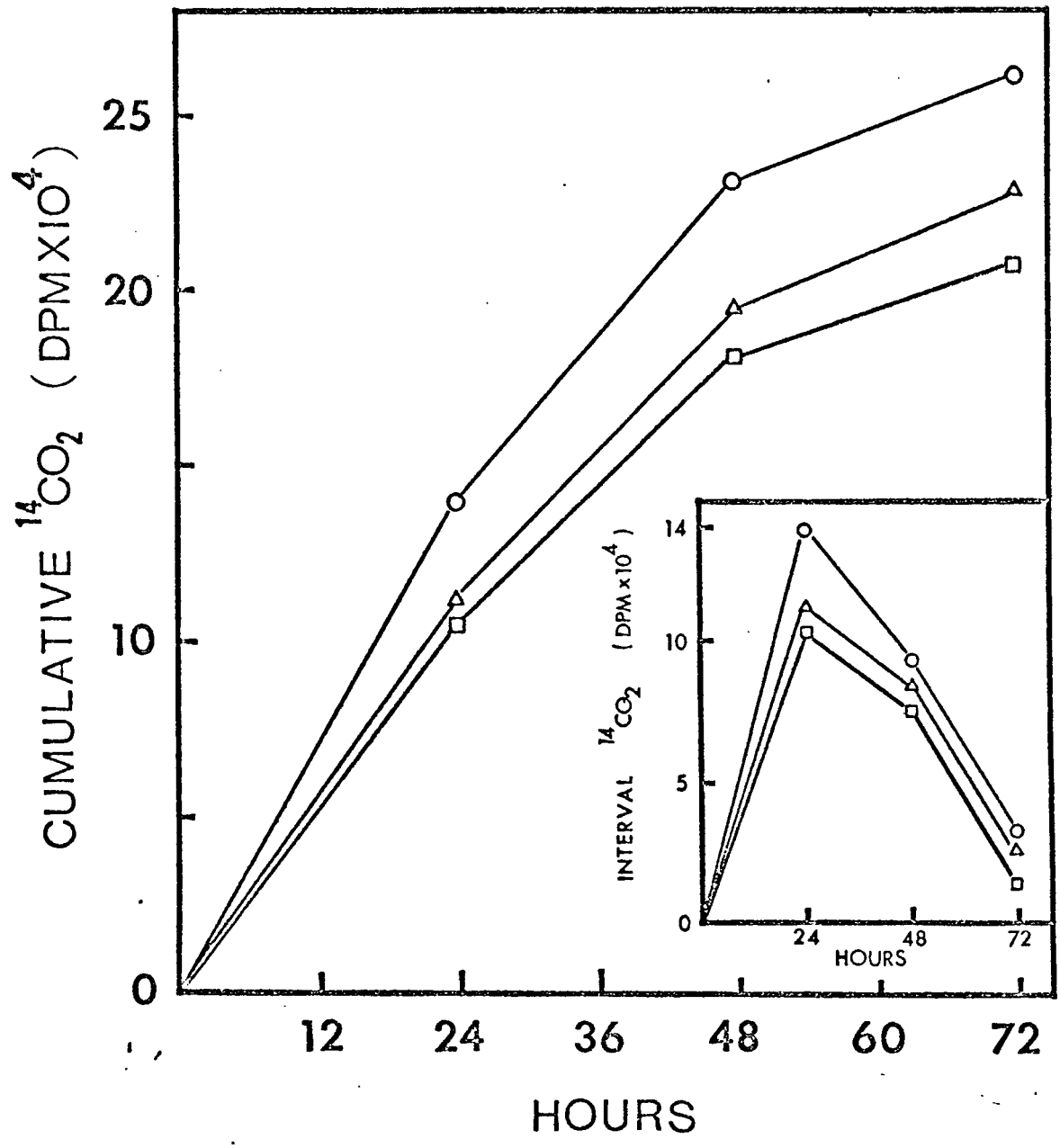
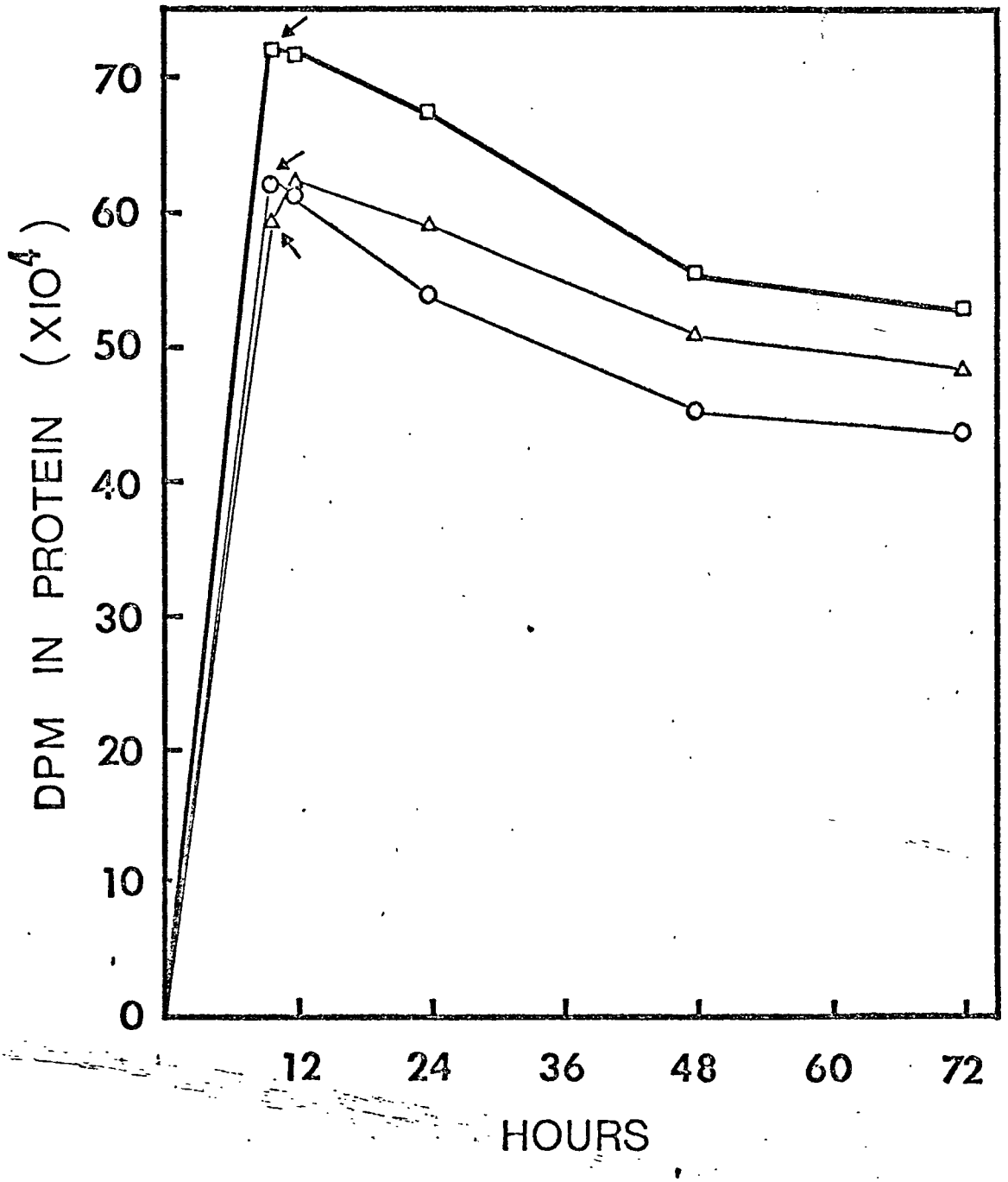

CUMULATIVE $^{14}CO_2$ (DPM X IO$^4$)
25
20
10
0
12
24
36
48
60
72
HOURS
INTERVAL $^{14}CO_2$ (DPM x 10$^4$)
14
10
5
0
24
48
72
HOURS

Fig. 12. Fate of $^{14}C$-labeled protein synthesized during encystment in basal salts. Mid-log phase myxamoebae-swarm cells grown in SD medium are transfered to 25 ml of basal salts containing different UL-$^{14}C$-amino acids and a $2x10^{-5}$ M concentration of the respective non-radioactive amino acid. The cell number is adjusted to $10^7$ cells/ml in each case. After 10 hours incubation ( as indicated by the arrows ), the labeled encysting myxamoebae are transfered back to 20 ml of non-radioactive BS for an additional 62 hours incubation. Two ml samples are collected at 0, 12, 24, 48, 72 hour intervals for analysis of DPM in TCA precipitates.

UL-$^{14}C$-valine (0.1 μCi/ml) O——O

UL-$^{14}C$-isoleucine (0.1 μCi/ml) △——△

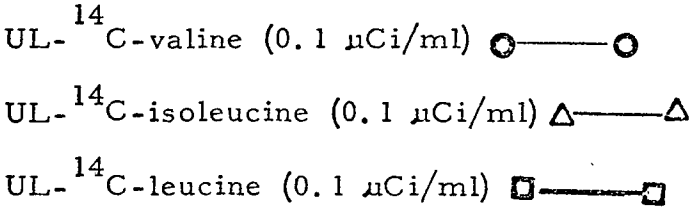
UL-$^{14}C$-leucine (0.1 μCi/ml) □——□


DPM IN PROTEIN (X10⁴)
70
60
50
40
30
20
10
0
12
24
36
48
60
72
HOURS

D) Fate of Radioactive "Growth State" Protein During Encystment

The $^{14}C$-myxamoebae-swarm cells labeled during growth by UL-$^{14}C$-amino acids are transfered to non-radioactive BS to induce encystment. There is a decrease of total DPM in the TCA precipitates and the UL-$^{14}C$-leucine labeled "growth state" protein is degraded less (Fig. 13), and $^{14}CO_2$ evolved is also less, than from cells labeled with UL-$^{14}C$-valine and UL-$^{14}C$-isoleucine, respectively (Fig. 14).

Fig. 13. Fate of "growth state" protein during encystment in basal salts. Radioactive myxamoebae-swarm cells grown in 25 ml each of SD medium containing different UL-$^{14}$C-amino acids for 48 hours are washed and incubated in non-radioactive BS for 72 hours. Two ml samples are collected at 24 hour intervals for analyses of DPM in TCA precipitates.



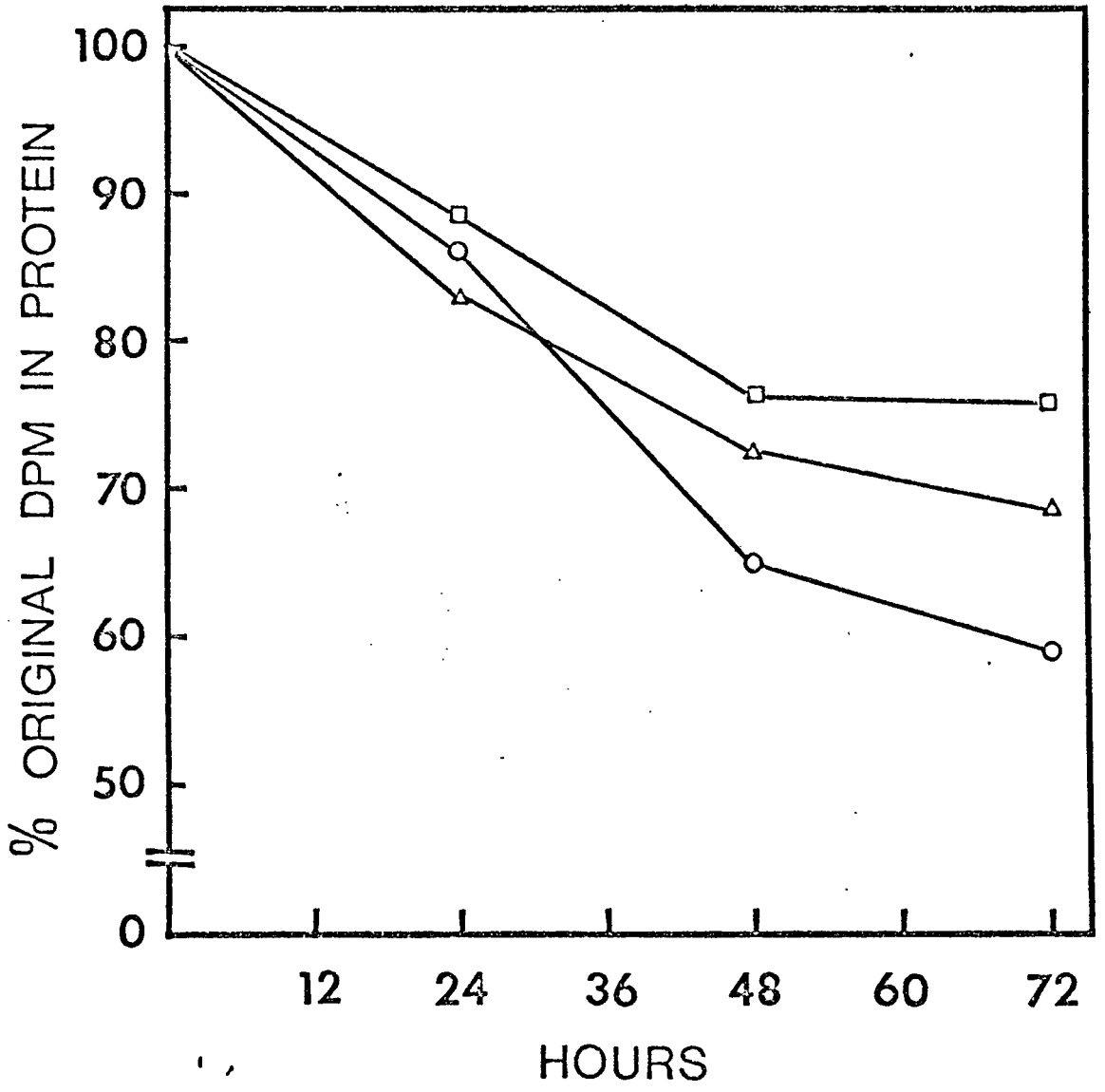
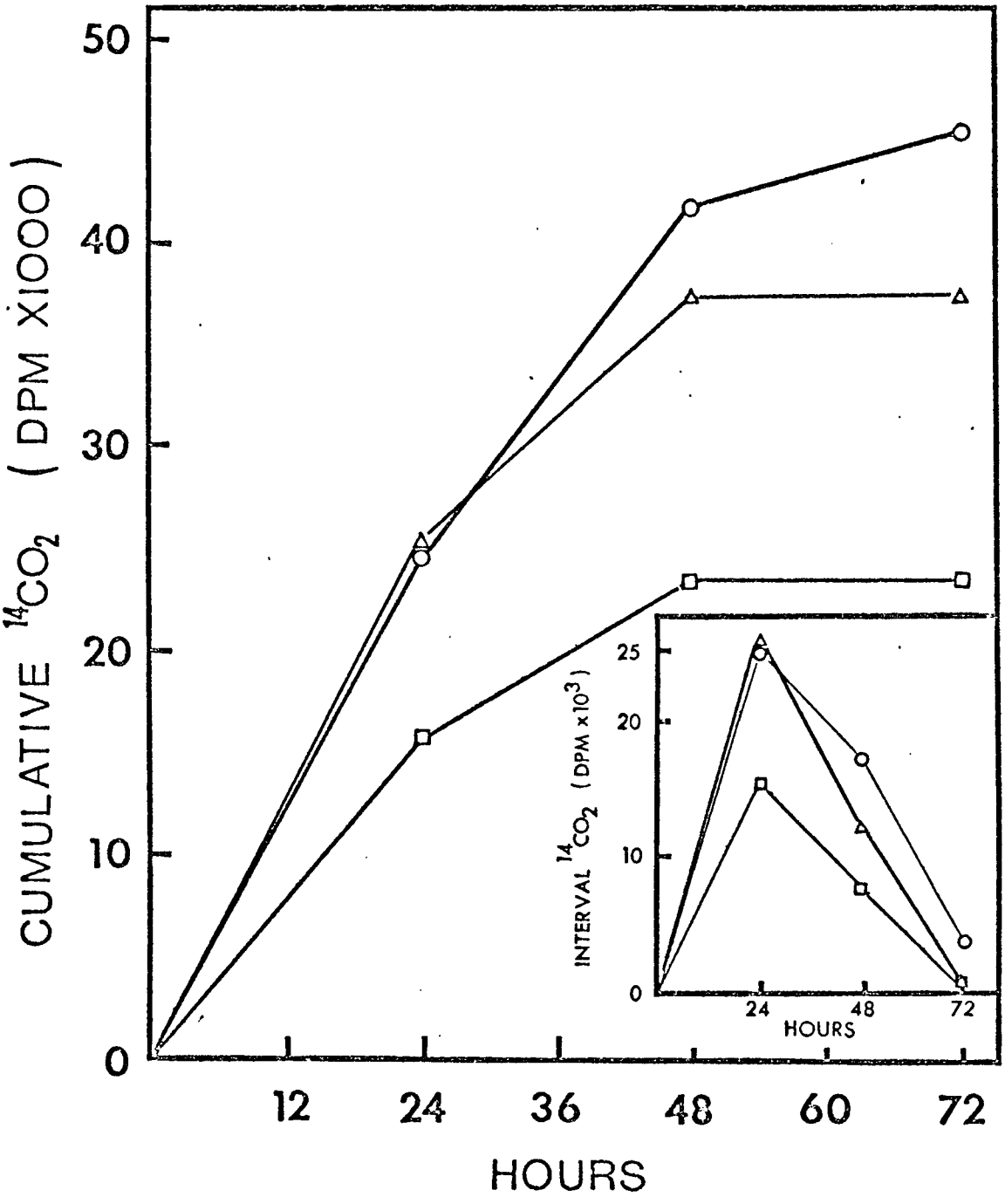

100
90
80
70
60
50
0
% ORIGINAL DPM IN PROTEIN
12
24
36
48
60
72
HOURS

Fig. 14. Cumulative and interval $^{14}CO_2$ evolved, from myxamoebae labeled by growth in $^{14}C$-amino acids, during encystment in basal salts. Radioactive myxamoebae-swarm cells grown in 25 ml each of SD media with different UL-$^{14}C$-amino acids for 48 hours are washed with non-radioactive BS and incubated in non-radioactive BS for 72 hours. The $^{14}CO_2$ evolved is collected in 1 ml of 20% KOH in a small test tube hanging inside the flask with a wire basket. KOH is changed every 24 hours. Radioactivity in the KOH samples is determined and the cumulative and interval $^{14}CO_2$ evolved are plotted in each case.

UL-$^{14}C$-valine (0.1 μCi/ml) O ——— O

UL-$^{14}C$-isoleucine (0.1 μCi/ml) △ ——— △

UL-$^{14}C$-leucine (0.1 μCi/ml) □ ——— □


CUMULATIVE $^{14}CO_2$ ( DPM X1000 )
50
40
30
20
10
0
12
24
36
48
60
72
HOURS
INTERVAL $^{14}CO_2$ ( DPM x$10^3$ )
25
20
10
0
24
48
72
HOURS

# DISCUSSION

The haploid cells of the Myxomycete Physarum flavicomum exist as both myxamoebae and swarm cells when grow in SD medium. However, upon transferring to non-nutrient basal salts solution, these cells lose their motile ability and form dormant microcysts with definite rigid cell walls, which enables them to withstand adverse environmental conditions. Encystment is a type of single cell differentiation (6) and such a system is suitable in many ways as model for the study of cellular differentiation. In the soil amoeba Acanthamoeba, and the cellular slime mold Dictyostelium, the differentiation processes have been relatively extensively explored (1, 39). Although the haploid stage of Physarum has been studied for a long time, most of the studies were carried out on solid media with either living or formalin-killed bacteria as a food source (14). Recently, Goodman (12) established a complex axenic liquid culture method for the haploid cells of Physarum polycephalum which enabled the performance of some modern biochemical studies on the haploid phase. Unfortunately, Goodman's medium is very complex and quite expensive. This drawback was overcome by Henney, Asgari and Henney (23) who adapted the haploid cells of Physarum flavicomum to a simple, inexpensive semi-defined medium, which is also the same medium used for the growth of the diploid cells. Recently, Henney and Asgari (21)

succeeded in growing the haploid cells in chemically defined minimal media.

During encystment of Physarum flavicomum, several major biochemical events are observed. First, the neutral hexoses are found to be rapidly degraded in the initial 12 hours. This suggests that carbohydrate, which is the major energy source for growth (24), is used for carbon and energy in the initial phase of differentiation. Lynch and Henney (34) found that during sclerotization of Physarum flavicomum carbohydrate metabolism is active only in the early phase. Henney and Maxey (24) later suggested that during sclerotization, carbohydrate is primarily used to form cellular structures such as the cell wall of the dormant cells.

Nucleic acid turnover during encystment is also studied. Although Sauer et al. (48) found 67% and 40% decrease in RNA and DNA, respectively, during diploid spherulation of Physarum polycephalum, there is a degradation of about 20% RNA and less than 10% DNA, during this haploid encystment of Physarum flavicomum. The net decrease of RNA may be a result of massive destruction of cytoplasmic materials during differentiation since condensation of cytoplasm is observed. However, the small amount of DNA degradation when compared with the diploid phase can be easily explained. The fact that the haploid cells are uninucleated and there is a greater than 90% conversion of vegetative cells to microcysts suggest that loss of nuclear materials

is not likely to occur. In contrast, the multinucleated plasmodia upon starvation distribute the nuclei to individual spherules and finally shrink to half of the original size (14). However, in encystment of Acanthamoeba, a considerable amount of DNA is lost (39). Neff et al. (39) suggests that the decrease may be due to the present of multinucleated amoebae in the stationary phase of growth and during encystment the breakdown of excess nuclei occurs.

One of the endproducts of encystment is the formation of microcyst walls. Early attempts to break the mature microcysts using a sonicator with glass beads or a nitrogen bomb did not give satisfactory results. Excessive heat generated by the sonicator usually destroys the microcyst walls. The only effectively method found for breaking the cells is a French press. Three passes through the press is sufficient for breakage and allows the recovery of large fragments of microcyst walls. However, in breaking the spore and spherule walls of Physarum polycephalum, McCormick et al. (36) used more than 20 passes. This indicates that the microcyst walls may be weaker than the walls from the other dormant stages of Physarum.

In order to purify cell walls, contaminating cytoplasmic materials must be removed. Most researchers (3,53,54) use differential centrifugation and exhaustive washing with distilled water. The salt-water-detergent cycles used by McCormick et al. (36)

for washing the spore and spherule walls was found to be useless in the purification of the microcyst walls. Early studies showed that upon the addition of salt (e.g., NaCl) to the walls, the dispersed milky-transparent walls clump together immediately to form dark-grey colored precipitates. Such clumping interfers with the removal of contaminating cytoplasmic materials. Furthermore, the use of SDS may result in the removal of some components of the walls such as the lipid and protein. However, by using distilled water, high purity microcyst walls are obtained after 15-20 washes.

Chemical analysis of the purified microcyst walls show that a galactosamine polymer is a part of the walls. This finding suggests that the galactosamine polymer may be a common constitutent of the dormant cells of Physarum. Further cell wall analyses on the other Myxomycetes may give more insight into the taxonomy and classification of the Myxomycetes. Galactosamine has been reported as a major part of the walls of other eucaryotes (2,5,55). For instance, 30% galactosamine is reported in the walls of the trichomycete, Amoebidium parasiticum. Recently, the cell walls of Aspergillus niger were studied and 20% galactosamine was reported. The present of a trace amount of the pentose D-ribose is not uncommon since Bartnicki-Garcia et al. (3) also found a small amount of D-ribose in the yeast walls of Mucor rouxii. Nield (41) also found 16.4% of non-hexosamine carbohydrate present as in

his early report on the spore walls of Physarum polycephalum. The present of neutral sugars in the microcyst walls is not likely to be cytoplasmic contaminants since different preparations of microcyst walls with extensive distilled water washes still indicated the present of an anthrone positive reacting material in the microcyst walls.

Amino acid analyses reveal a total of 17 amino acids with traces of methionine. Performic acid hydrolysis on the microcyst walls shows the present of small amounts of cysteic acid. The fact that these 17 amino acids are present in both preparations and the mole percent in each case is relatively close, strongly suggests that the amino acids are not cytoplasmic contaminations but are actual components of the walls.

It has been found that during differentiation of Physarum polycephalum and P. flavicomum, an increase in the content of lipid is observed (51). This lipid probably serves as a storage material formed during the metabolism of carbohydrate (50) and may also serve as component of the membrane since Cheung et al. (8) reported an increase of membrane during sclerotization of P. flavicomum. Also, a considerable amount of lipid is found in the microcyst walls of P. flavicomum. Whether this lipid is bound or free has not been determined. It is not uncommon to find lipid associated with walls and 21% lipid is found in Polysphondylium pallidum microcyst walls (53). Lipid and protein are

major components of cell membranes and it is questionable whether the present of these two components are directly from the cell walls or the adherent membrane. However, staining of the purified walls with a specific lipid dye, O red oil, did not show any red membrane attached to the microcyst walls as observed by light microscopy.

Phosphate has been known to be part of the cell walls of both procaryote and eucaryote cells (3, 31, 46, 53). In Mucor rouxii about 20% phosphate is found (3), although Harold (20) reported the artifact of inorganic phosphate attached to cell walls of Neurospora crassa. The presence of phosphate may serve as linkages in the cell wall such as in Staphylococcus lactis in which teichoic acid and mucopeptide are linked together by phosphodiester bond (46). Phosphate may also associate with hexosamine and muramic acid in the wall (32). In some gram-positive bacteria, such as Streptococcus sp., Micrococcus leisodeikticus, Staphylococcus aureus and Mycobacterium butyricum, a considerable amount of muramic acid-6-phosphate is found. The authors suggest that the phosphorylated muramic acid residues may serve as a bridge between the mucopeptide polymer and the cell wall polysaccharide or teichoic acid in the gram-positive bacterial cell walls. Bartnicki-Garcia et al. (3) also suggest that phosphate present in the Mucor rouxii walls may be associated with lipid and nucleic acid detected in the walls.

These data on the microcyst walls indicate mark differences between them and the spore walls as well as the spherule walls of a closely related species of Physarum, except that a galactosamine polymer is the common component of the wall. Since the two species are both grown and differentiated in quite a similar fashion, whether these closely related species should have such great differences remains unanswered. Since the haploid cells can now be grown in the same medium as the diploid cells of Physarum flavicomum the compositions of these two types of walls can now be studied and compared under the same conditions.

The process of differentiation has been a controversial topic for a long time. The actual mechanism for this process still remains unknown. In the cellular slime molds, extensive studies have been conducted in order to explain the process. Sussman and co-workers (52) studied the three key enzymes in the morphogenesis of Dictyostelium discoideum (trehalose-6-P synthetase, UDPG pyrophosphorylase, UDP-galactose transferase). When cycloheximide is added before the usual period of accumulation of these enzymes, no enzyme accumulation is observed. However, if this drug is added during the time of enzyme accumulation, it inhibits further accumulation and prevents the usual disappearance of such enzymes after the period of accumulation. The accumulation of each of

these three enzymes was also shown to be affected by RNA synthesis. In the case of UDPG pyrophosphorylase which accumulates between 11-12 and 20 hours after induction of morphogenesis, addition of actinomycin D before 5 hours prevents any increases in enzyme, but the addition after 12 hours allowed a normal enzyme accumulation as in the control. From these data, Sussman postulated that enzyme accumulation results from an induction of enzyme synthesis which is dependent upon specific periods of transcription and translation. However, Gustafson et al. (16) disagree with this postulate. They isolated and purified UDPG pyrophosphorylase of the same organism from both vegetative amoebae and culmination stage cells. By using $^{35}S$-methionine incorporation into the cells and comparing the radioactive in enzyme protein and acid-insoluble protein, they found that during differentiation, enzyme synthesis is at a rate similar to the vegetative cells but the rate of enzyme degradation is lower. Also, by using specific UDPG pyrophosphorylase antisera obtained from rabbits, they demonstrated that the vegetative cell enzyme is twice as susceptible to the antisera as the culmination stage cell enzyme. They concluded that the enzyme accumulation observed during differentiation is mainly due to the decrease in enzyme degradation. In procaryotes, gene expression during differentiation is also described (17). For instance, in Bacillus subtilis,

both new protein and RNA are found to occur during sporulation. Halvorson (17) described that sporulation is a genetic control system where the vegetative genome is being depressed while, on the other hand, a spore genome is being activated.

In the Myxomycetes, especially in the genus Physarum, diploid differentiation (sclerotization) is being heavily studied since the establishment of axenic liquid media (12, 23). Recently, Henney and Maxey (24) studied the factors that may be responsible for the initiation of sclerotization. Results indicate that instead of complete starvation, an imbalance of nutrients may be responsible for initiating the process. Glucose, the major carbon source for growth, when present in the sclerotization medium does not affect the process. On the other hand, a slight enhancement of cell wall formation is observed. However, casein hydrolysate and ammonium nitrate are found to delay sclerotization.

In encystment of Physarum flavicomum different supplements are tried for their effects. Since casein hydrolysate contain 18 different amino acids, the rate of encystment in the presence of these amino acids in BS are tested. The results indicate that the aliphatic amino acids are the only group that delays encystment in a similar way as the casein hydrolysate. Further elucidation shows that L-valine, L-isoleucine and L-leucine are the only amino acids that cause marked delay in the rate of encystment. The present of

glucose and ammonium nitrate in the basal salts solution do not affect the rate of encystment and light microscopy does not indicate a more distinct cell wall formation. Since the cell walls have a galactosamine polymer, these nutrients may be simply incorporated instead of catabolized by the cells and the fact that the pH of these media do not differ from the control media supports such a contention. However, when different amino acids are present in the basal salts during encystment, a slightly higher pH in these media than the control is observed after 72 hours incubation.

Protein degradation has been commonly observed during diploid differentiation of Physarum (3, 23). During encystment, about 40% of protein is degraded consistently throughout the 72 hour process. Encystment of $^{14}C$-myxamoebae in the presence of different supplements show that the "growth state" protein, under the influence of amino acids (casein hydrolysate, L-valine, L-isoleucine and L-leucine), is conserved and degraded slower than the control.

In order to further understand these events, the rate of protein synthesis and degradation during growth and differentiation should be compared. One of the early experiments was done in 1958 when Halvorson (18, 19) used Saccharomyces cerevisiae and found that during the resting phase, the yeast cells breakdown protein 23 times more than in the growth phase. Such protein degradation is suggested to be associated with the replenishing of the intracellular pool

when an exogenous source of amino acids are not available.

Similar experiments performed on encystment indicates that amino acids are taken up both during growth and during encystment. Also, the rate of degradation of "growth state" proteins is less than the proteins produced during encystment. These data strongly suggest that a possible shift of metabolic pattern during encystment may occur with the cells using amino acids as the prefered energy source. In plasmodia of Physarum flavicomum, this event has been demonstrated. Lynch and Henney (35) using a simpler medium BTC and UL-$^{14}$C-protein hydrolysate found that only 2.6% of the administered isotope is evolved as $^{14}CO_2$ after 16 hours incubation by the growing plasmodia. However, Henney and Maxey (24) reported that 45% of the total cellular DPM of the plasmodia labeled with UL-$^{14}$C-protein hydrolysate in the BTC medium is evolved as $^{14}CO_2$ after 48 hours incubation in the non-radioactive BS. These results strongly suggest a shift of metabolic pattern during diploid differentiation of Physarum flavicomum.

The fact that the $^{14}$C-leucine labeled "growth state" proteins are degraded little during encystment suggests that the cells may incorporate this amino acid and form "differentiation state" protein that are conserved during encystment. Since the rate of encystment has been demonstrated to parallel protein degradation, these newly formed proteins incorporated from the amino acids may serve as a "supp-

ressor(s) of differentiation".

Proteases are the common class of enzymes that are associated with protein degradation. Preliminary results show that leucine aminopeptidase activity increases about 2 fold during encystment (unpublished data). Recently, Hoffmann and Hutterman (26) suggest that the increase in activity of aminopeptidase may compensate for the loss of protein during sclerotization.

Amino acids have been known to affect differentiation in other organisms. In Bacillus cereus, a series of L-amino acids are found to affect germination of spores (57). When L-alanine is added to the medium, germination is accelerated. The author suggests that the metabolism of these L-amino acids provide the energy that "trigger" germination and these compounds may serve as allosteric effectors on other proteins. Proteolytic enzymes are also affected by some amino acids. For instance, in extracts of ovarian cancer cells, amino acids are found to inhibit the activity of the hydrolytic enzyme alkaline phosphatase (9). L-Leucine (0.5 mM) is found to inhibit about 45% of the normal activity (using 0.5 mM D-leucine as standard). L-Phenylalanine and L-tryptophan (10) are also found to suppress such enzyme activity.

Recently, an alkaline phosphatase in E. coli (30, 31) is found to co-regulate with an protease, aminoendopeptidase (using L-alanine-p-nitroanilide as substrate). Three proteins near the cell surface has been found to co-regulate with this alkaline phosphatase (38).

However, no enzymic activity has been attributed to any of these proteins. Thus, the authors cannot conclude that any of these proteins is actually the aminoendopeptidase *per se*. These reports, indicate that L-amino acids may have a remote control mechanism on the activity of a protein(s) and these protein(s) in turn controls the proteolytic activity of the organism which results in the control of protein degradation during differentiation.

Chemical analysis on the purified microcyst walls of *P. flavicomum* revealed that this is a rather complex structure compared to the reported (36) single D-galactosamine polymer in the spore walls and spherule walls of *P. polycephalum*. The last part of this thesis indicates that a shift in metabolic pattern during encystment may occur and the initiation of the process is due to an imbalance of nutrients rather than the starvation of the cells *per se*. This paper serves as a beginning in the biochemical study of encystment in the Myxomycetes under defined medium and under controlled conditions.

# SUMMARY

Encystment of Physarum flavicomum variety 1 in basal salts solution has been studied. More than 90% encystment is observed within 72 hours incubation. Biochemical analyses of the intracellular protein, total hexose, RNA, and DNA are performed during encystment. Isolated and purified microcyst walls are found to contain D-galactosamine, D-glucose, D-galactose and D-ribose. Lipid and protein are the major components and a small amount of inorganic phosphorus are present in these walls. Protein sources, for instance, casein hydrolysate, L-leucine, L-isoleucine and L-valine are found to delay encystment. Radioactive experiments using UL-$^{14}$C-amino acids are used to compare the rate of protein degradation during growth and encystment. Possible role of protein degradation during this differentiation is discussed.

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