DEVELOPMENTAL PHYSIOLOGY OF THE ZOOSPORE OF BLASTOCLADIELLA EMERSONII

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ABSTRACT

DEVELOPMENTAL PHYSIOLOGY OF THE ZOOSPORE OF BLASTOCLADIELLA EMERSONII

By

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The zoospore of Blastocladiella emersonii represents a stage in the life cycle which does not grow or carry on synthesis of cellular components, but does respire actively for the energy necessary for movement and maintenance. A study of the physiology of these zoospores was begun both to provide a reference point for the changes which take place when these cells encyst and to obtain a better understanding of a cell which apparently only carries on catabolic metabolism to provide the energy it requires to survive until it can begin the growth phase.

In order to study physiological changes associated with zoospores, existing liquid culture methods were modified and refined to allow production of large quantities of zoospores synchronously. In the final system PYG-P was used as the liquid medium and the plants were made to differentiate into sporangia by induction with a dilute inorganic salt solution.

Methods for keeping zoospores from encysting or making them encyst synchronously were also developed. In two of the incubation solutions with a short chill period, there was an increasing self-inhibition of encystment with increasing population densities. In one (MOPS-calcium), however, this effect was reversed, and the presence of calcium in an uncomplexed form was thought to be responsible.

The rate of oxygen uptake increased more than twofold during encystment (from a Q_{O_2} (cell) of 8-9 in nonencysted spores to 19-21 in encysted cells). Concomitant with this increase was a sharp decrease in the polysaccharide content (90% in 20 minutes).

Zoospores could be prevented from encysting by incubating them without a chill period in an inorganic salt solution containing calcium. The polysaccharide, lipid and protein content of these cells decreased with endogenous respiration while the nucleic acid and cell counts remained relatively constant. The rate of protein loss was about three times the rate of disappearance of the other two substances and was further documented by the accumulation of NH_3-N in the medium in an amount corresponding to the protein-N lost. An R.Q. of 0.91 also supported these results. Fine structure changes of zoospores incubated under endogenous nutrient conditions shower fewer lipid particles and a thinning of another organelle, the sb matrix, with increasing spore age. This was considered indirect evidence for localization of the lipid and protein reserves in these organelles, respectively.

The rate at which the exogenous substrates, glucose and glutamate were taken up by zoospores was dependent on the concentration of these substances. Below 1 mM, the uptake rate was very low, but above 1 mM it increased sharply three to tenfold. The rate of disappearance of the internal polysaccharide content also varied with the amount present in the cell; the rate doubled when the level of this constituent exceeded 2.0 picograms/spore.

The relative energy consumption required for flagellar movement was estimated from the rate of oxygen consumption of deflagellated versus

flagellated zoospores. A drop of 15% in the Q_{O_2} indicated that a minimum of 15% of the energy production of the cell was required for flagellar activity. Other energy requirements of the cell are also discussed.

DEVELOPMENTAL PHYSIOLOGY OF THE ZOOSPORE OF *BLASTOCLADIELLA EMERSONII*

By

Keller Francis Suberkropp

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LIST OF SYMBOLS AND ABBREVIATIONS

OC	Ordinary colorless
RS	Resistant Sporangia
S	Salt solution used as incubation medium for zoospores
W	Number of times zoospores were washed by centrifugation
T	Length of time (hours) zoospores given chill period
T'	Length of time (hours) zoospores incubated at incubation temperature
P	Population density
%NE	Percent nonencysted
%E	Percent encysted; in general, %E=100-%NE
Q_{O_2} (cell)	ul O_2 hr^{-1} $(10^7$ cells$)^{-1}$
R.Q.	Respiratory quotient
pg	Picograms (10^{-12} grams)
ADP	Adenosine 5'-diphosphate
BP	Blastocladiella polysaccharide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether) N, N'-tetracetic acid
MOPS	Morpholino propane sulfonic acid
NADH	Reduced nicotinamide adenine dinucleotide
RNA	Ribonucleic acid
TCA	Trichloroacetic acid
Tris	Trishydroxymethylaminomethane

INTRODUCTION

The aquatic Phycomycete, Blastocladiella emersonii, has been used as a vehicle for the investigation of differentiation at a biochemical level since it was isolated from nature and brought into the laboratory in 1949 (Cantino and Hyatt, 1953). Much work has been done on the physiological and enzymological bases which cause plants of this fungus to develop along either of two basic pathways. One of these leads to the formation of thin walled, ordinary colorless (OC) sporangia (see Figure 1) which differentiate and release zoospores immediately after the growth phase is completed. The other leads to the formation of thick walled, brown, resistant sporangia (RS) which can undergo a period of dormancy before they differentiate and release zoospores. For reviews on the nature and cause of these developmental shifts, see Cantino and Lovett, (1964) and Cantino, (1966).

Recently a number of studies have dealt with encystment and subsequent germination of the zoospore of B. emersonii, the first developmental changes common to both pathways. These studies have examined: (i) morphological changes at both the electron and light microscope levels (Truesdell and Cantino, 1971; Soll et al., 1969); (ii) the ionic requirements for encystment (Truesdell and Cantino, 1971; Soll et al., 1969); and (iii) initiation of synthesis of protein and ribonucleic acid (Lovett, 1968; Soll and Sonneborn, 1971). There have also been a number of publications on the fine structure of the zoospore

Figure 1. The OC Life Cycle of Blastocladiella emersonii. Stages are not drawn to scale but as to their relative importance in this thesis; therefore, A is on a larger scale than B and C, which are on a larger scale than D and E. A: zoospore showing gamma particle (G), lipid particle (L), backing membrane (BM), sb matrix (SB), banded rootlet (R), flagellum (F), kinetosome (K), mitochondrion (M), nuclear cap (NC), nucleus (N), and nucleolus (NU). B: encysted cell. C: germinating cell. D: papilla formation in OC plant. E: discharge in OC plant.

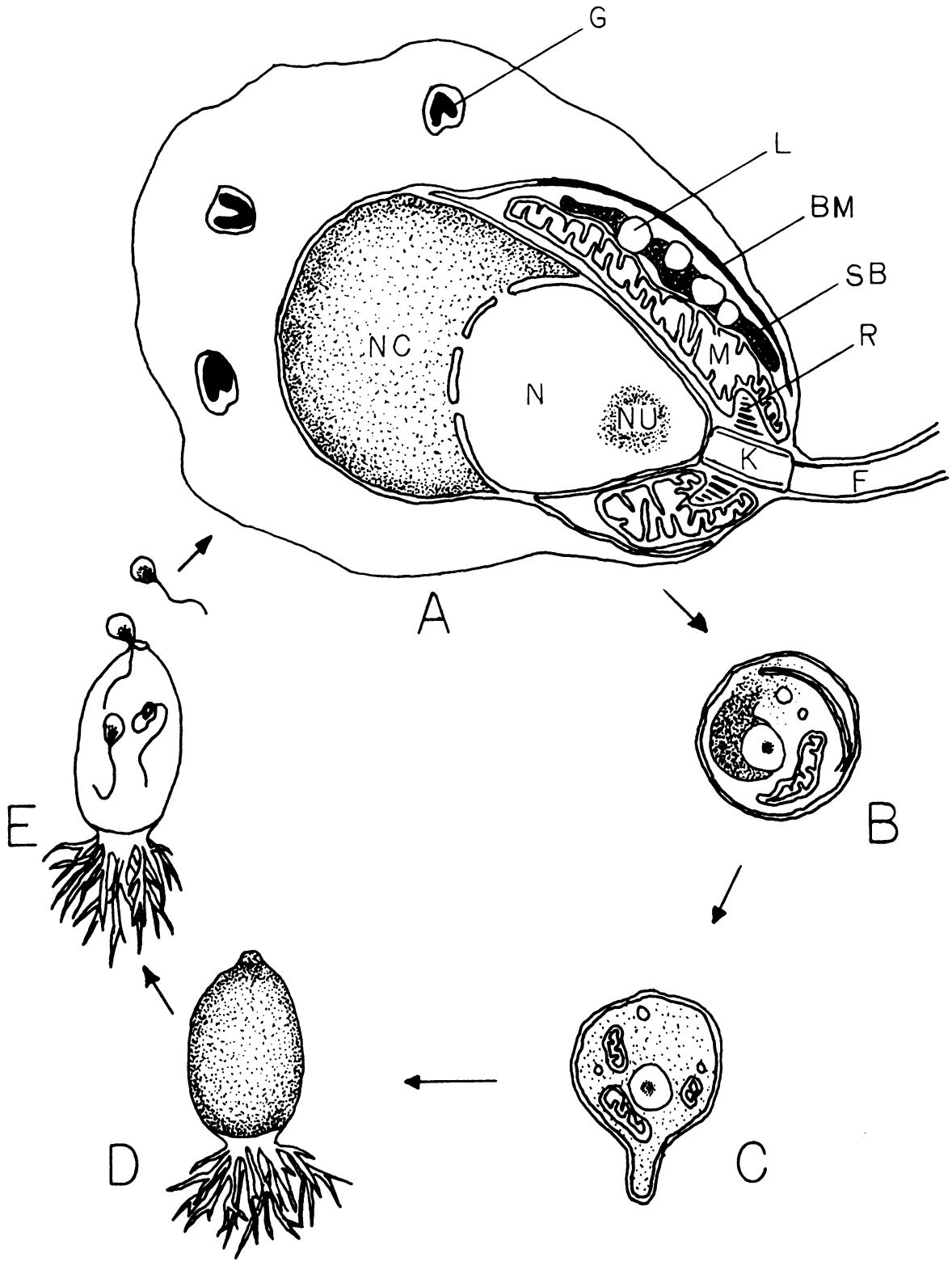

Figure 1. The OC Life Cycle of <u>Blastocladiella emersonii</u>.

itself (see Truesdell and Cantino, 1971, and references therein).

In order to introduce this thesis, a brief description of those aspects of zoospore structure and changes during encystment and germination which are of primary concern to this work follows.

The zoospore of B. emersonii (Figure 1A) has a single posterior whiplash flagellum. It possesses no cell wall and is therefore separated from the environment only by a plasma membrane. The organelles inside the cell are highly ordered and consist of: (i) the nuclear apparatus with its nucleus, nucleolus and nuclear cap (which contains most of the ribosomes in an inactive state); (ii) the gamma particles, small DNA containing organelles in the cytoplasm; (iii) the side body, consisting of a single mitochondrion, lipid particles next to it, sb matrix (the function and composition of which is unknown) in which the lipid is embedded, and the backing membrane which surrounds the side body (Cantino and Truesdell, 1970); and (iv) the banded rootlet, positioned in channels of the mitochondrion and next to the kinetosome, at the base of the nuclear apparatus.

Encystment of zoospores occurs when they become spherical, rotate the nuclear apparatus, withdraw the flagellum and lay down a cell wall very rapidly. Intracellular changes which follow include breakdown of the gamma particles, dispersion of both the ribosomes of the nuclear cap and the lipid particles of the side body into the cytoplasm, disappearance of the axoneme inside the cell, and the extension and furrowing of the mitochondrion (Figure 1B).

Germination has been arbitrarily defined as the time when the germ tube is formed and the cell enters the growth phase and begins synthesizing protein and nucleic acid (Figure 1C).

The work reported here began as a general study of the physiological changes that take place during encystment. In order to study this, however, it was felt that a reference point had to be established, i.e. the physiological changes which occur in the nonencysted zoospores first had to be thoroughly examined. As will be seen, the latter turned out to be the primary goal reached during the course of this study. Except for the data in Results, I, B, on oxygen uptake and endogenous polysaccharide utilization during encystment, this work deals mainly with the physiology of nonencysted zoospores of B. emersonii.

Although the zoospores of B. emersonii, and quite likely those of most other water molds, are very different structurally and, in some aspects, physiologically, from the spores of other fungi, they are similar in some ways. They serve as propagules and do not grow. They represent a stage in the life cycle in which biosynthetic activity has been shut off and are, therefore, dormant in this sense (Sussman, 1965; Truesdell and Cantino, 1971). Their dissimilarities in structure with other fungus spores probably also account for some of the physiological differences. The lack of a cell wall requires that the cell expend energy to maintain osmotic balance with the medium, and the motile nature of the cell also requires energy output. Therefore, the cell must respire at a relatively high rate and metabolize nutrients to maintain these energy levels. In this respect, a study of the physiology of nonencysted zoospores offers more than just the needed reference point for future studies on the physiological changes which occur during encystment. It offers the chance to study a cell which is unique in several of its activities, a cell which, using either endogenous

reserves or exogenous nutrients, seemingly carries on only catabolic metabolism to provide the energy for maintenance and motility without the complications of biosynthesis and growth.

MATERIALS AND METHODS

I. Manipulations of the Organism.

A.) Agar Cultures. Zoospores produced for some experimental work and as inocula for liquid cultures were routinely derived from OC plants grown at 22 C on Difco Cantino PYG Agar in the dark in 10 cm plastic Petri plates at densities of 1-3 x 10^5 plants/plate. Under these conditions, the first generation OC plants discharged at 22 to 26 hours.

Because continuous subculturing of OC plants can cause variation in some characteristics (Shaw and Cantino, 1969), RS from stock cultures were germinated every 1 to 3 months to provide a fresh OC line. (see Lovett, 1967, for a description of procedures).

B.) Liquid Cultures. Two liquid media were used for growth of synchronous single generation OC plants: (i) PYG-P; 1.25 g Peptone, 1.25 g Yeast Extract, 1.5 g Glucose, and 2.5 mmoles each of KH_2PO_4 and K_2HPO_4 per liter water (Goldstein and Cantino, 1962); (ii) PYG-PC; 1.25 g Peptone, 1.25 g Yeast Extract, 3.0 g glucose, 11 mmoles Na phosphate, pH 6.7, and 3 mmoles citric acid per liter water (Cantino and Goldstein, 1962). For preparation of inocula, PYG plates of OC plants were flooded after discharge had begun with ca. 5 ml water and the resulting spore suspension passed through Whatman no. 4 filter paper into a flask in ice. The spore suspension was chilled (generally 15 to 20 minutes) until the zoospore density had been determined with a Coulter Counter and an appropriate amount pipetted into the liquid medium.

The liquid cultures were prepared with either 1.2 liters of medium in a 2.0 liter cotton plugged Erlenmeyer flask with 2 aeration tubes (aeration rate = 6 liters air/minute) and a sampling tube, or 4.5 liters of medium in a 6.0 liter Florence flask with 3 aeration tubes (aeration rate = 10 liters air/minute) and a sampling tube. Dow Antifoam A was sprayed on the aeration tubes before autoclaving. The culture flasks and media were always preincubated in the constant temperature bath and aerated for 1-2 hours before inoculation. Cultures, grown in light, were illuminated with 350-450 foot candles.

For production of zoospores from these cultures induction was necessary. Inducing media were: (i) Calcium-citrate solution; 3 mM Na_3 citrate, 1 mM $CaCl_2$, 7 mM NaCl and enough HCl to lower the pH to 6.8; (ii) Modified $\frac{1}{2}$ DS; 0.025 mM each of $MgSO_4$ and $CaCl_2$, and 0.25 mM each of KH_2PO_4 and K_2HPO_4; (iii) MOPS-calcium solution; 0.5 mM MOPS, pH 6.8, and 0.1 mM $CaCl_2$. The induction procedure was similar to that described previously (Murphy and Lovett, 1966) but with modification. Aeration was stopped and the plants allowed to settle. The spent medium was removed by suction (4.3 liter from the 4.5 liter cultures and 1.0 liter from the 1.2 liter cultures). Inducing medium was added to the plants (3.5 liter and 1.0 liter respectively), aeration resumed for 5 minutes, then aeration stopped and the medium removed as before. Finally a second batch of inducing medium was added (1.0 liter and 0.5 liter respectively) and aeration resumed until the end of the generation time, i.e. until maximum discharge and harvest. In this manner 97-98% of the spent PYG was removed and the plants were placed in a medium very low in nutrients.

After induction, samples of the plants were examined microscopically.

Two characteristics (papilla formation and discharge) used by Murphy and Lovett, (1966) were followed quantitatively to determine the degree of differentiation and its synchrony; at intervals, 100-200 plants were scored for each of these characteristics.

C.) Harvesting. When the populations in liquid cultures had reached their maximum discharge percentage (generally 90-100%), the suspension of spores and plants was passed through Sargeant #500 filter paper to remove plant walls, undischarged plants and any encysted spores that might be present. The spores were then counted with the Coulter Counter and washed and concentrated by centrifugation. Since the volume of spore suspension was large (0.7-1.2 liter), washing consisted of successive centrifugation of four x 35 ml aliquots at 1000 x g for 3 minutes. After centrifugation, the pellets were immediately resuspended in small volumes of incubation solution and pooled to help prevent damage. The second centrifugation, also 1000 x g for 3 minutes, was done on the combined spores since the total volume had been reduced to ca. 100 ml. Thus each spore only received two - 1000 x g centrifugations of 3 minutes each. The entire procedure was done at 22-24 C to prevent encystment and took 1-1½ hours. Microscopic examination of zoospores after this treatment showed that damage was minimal; approximately 5-15% of the spores were deflagellated (for a summary of procedures used in harvesting spores from plates see Table 1 in Results, I).

D.) Zoospore Incubations. Zoospore suspensions produced on plates or in liquid cultures were incubated in several systems; (i) for determination of percent nonencystment (%NE) only, 3 ml were incubated in 25 ml Erlenmyer flasks in an Eberbach water bath reciprocating shaker (stroke, 2.5 cm; 70 cycles/minute); (ii) for determination of oxygen

uptake with an oxygen electrode and the corresponding %NE, 5 ml were incubated in 15 ml vials over a magnetic stirrer and immersed in fluid from a constant temperature circulator; (iii) for manometric determination of oxygen uptake, 2-3 ml were incubated in a Warburg apparatus; and (iv) for determination of other physiological changes and corresponding %NE, 25-800 ml were incubated in jacketed Bellco Spinner Flasks of appropriate size with temperature controlled by a circulator. Suspensions were agitated by both magnetic stirring and aeration (0.6 liters air/minute for 25 to 50 ml and 2 liters air/minute for 800 ml).

E.) Spore Counts. For determination of %NE, 0.5 ml of spore suspension were removed from the incubation mixture, fixed with ice-cold 0.5 ml 4% glutaraldehyde (J. T. Baker Chemical Company) in 5 mM MOPS, pH 6.8, and kept cold until scored in an Improved Neubauer Levy Ultraplane Counting Chamber on a Wild phase microscope for nonencysted cells ml. These counts were compared with values obtained similarly for zero time to calculate %NE.

Live cell counts for determination of inocula sizes, yields of zoospores, and population densities were made with a model B Coulter Counter. The electrolyte solution for dilution of cells contained 10 mM KCl and 10 mM NaCl. The size of the aperature was 100 μ with settings of 20 and off scale for lower and upper thresholds respectively. Reciprocals of aperature current and amplification were 0.707 and 2, respectively.

II. Oxygen Uptake.

A.) Oxygen Electrode. Oxygen uptake was measured with a Clark type oxygen electrode. Both the Beckman Polarographic O_2 sensor (#39065) and the YSI O_2 sensor (Model 5331) were used. Current changes across the electrode were followed with a Heath Servo Recorder (EUW-20A). Procedures for calibration and use of O_2 electrode were taken from Estabrook, (1967); Davies, (1962); and Severinghaus, (1968). Q_{O_2} was expressed here as a value based on 10^7 cells instead of mg dry weight. Therefore, Q_{O_2} (cell) = ul O_2 hr^{-1} $(10^7 \text{ cells})^{-1}$. Additions of substances to O_2 electrode chambers were made through a port down the side of the chamber with a Teflon needle attached to a 1 ml syringe. Generally, 0.05 ml of concentrated solution were added to 5 ml of spore suspension.

B.) Warburg. A Precision 7 unit Warburg Apparatus was also used for oxygen uptake measurements. Methods were taken from Umbreit et al., (1964) including use of the Pardee CO_2 buffer and indirect determination of CO_2 for R.Q. determinations.

III. Microscopy.

A.) Electron Microscopy. Zoospores were fixed with glutaraldehyde and osmium tetroxide ("Fixation I"), dehydrated, embedded, and sectioned according to Truesdell and Cantino, (1970). Pictures were taken on a Phillips 100 Electron Microscope.

B.) Light Microscopy. Light microscopic observations were made with a Wild M20 phase microscope using a Bausch and Lomb ribbon filament

light source fitted with an infrared filter to reduce the heat transmitted to the speciment. The microscope was fitted with a Kodak Pony II, 35 mm camera back and photomicrographs were taken with Kodak High Contrast Copy Panchromatic film at exposure of 0.1 second for 20x objective and 0.5 second for 40x objective.

IV. Other Analytical Procedures.

A.) Dry Weight. Spore suspensions were removed from the incubation mixture and dried to constant weight at 90 C in a vacuum oven, along with fresh incubation medium as the blank.

B.) Nucleic Acid. Nucleic acids were precipitated from spore homogenates with ice-cold 10% TCA, washed twice by centrifugation with cold 5% TCA, then separated from protein by heating with 5% TCA at 90-95 C for 20 minutes. Nucleic acid was estimated with the orcinol reagent (Schneider, 1957), purified yeast nucleic acid (Calbiochem), being used as the standard.

C.) Protein. Protein was precipitated with ice-cold 10% TCA, washed twice by centrifugation with cold 5% TCA, and dissolved in 1 ml 1N NaOH at 90-100 C for 10 minutes. Protein was estimated with the Folin-Ciocalteu method of Lowry et al., (1951), using similarly treated bovine serum albumin (Nutritional Biochem, Fraction V) as the standard.

D.) Nitrogen. Free NH_3-N was determined by direct Nesslerization with commercial (Harleco dry pack) Folin and Wu (1919) reagent. Total N was estimated by wet digestion with H_2SO_4 and H_2O_2 (Umbreit et al., 1957) followed by Nesslerization.

E.) Blastocladiella Polysaccharide (BP). BP was extracted according to Cantino and Goldstein, (1961) with cold 10% TCA and the precipitate washed twice with cold 5% TCA. It was precipitated from the combined extracts with 95% ethanol (final concentration, 55-65%) at -18 C overnight, generally reprecipitated once, and hydrolyzed 2 hours in a steam beath in 0.6 N HCl. After removing excess HCl, the glucose liberated was estimated with the modified Glucostat (Worthington Biochemicals) method of Washko and Rice, (1961).

F.) Lactic Acid. Analyses for lactic acid were made with the microdiffusion method of Ryan, (1958).

G.) Lipid. Lipid was extracted from zoospores by the Folch et al., (1957) procedure, including modifications (Radin, 1969). Pellets of zoospores were sonicated on a Branson Sonicator for 30 seconds at setting 5 with the macrotip. Chloroform-methanol (1:1) was added, the suspension sonicated again, centrifuged, washed once (ca. 1000 x g, 5 minutes), and then brought to a chloroform-methanol ratio of 2:1. One fifth volume of 0.04% $CaCl_2$ was added, the suspension shaken thoroughly, centrifuged to separate phases, and the lower phase kept as the total lipid fraction. The surface of the lower phase was washed twice, evaporated to dryness at 60 C under N_2, the residue dissolved in chloroform, and used as the lipid extract.

Total lipid was estimated by drying to constant weight at 80 C in a vacuum oven, or by the dichromate method (Bragdon, 1951) for total organic material with palmitic acid (Sigma) as a standard. Neutral lipid and phospholipid fractions were separated by adding eight-tenths of the extract to 1 g of silicic acid (Mallinckrodt, suitable for chromatography) and extracting three times with chloroform

for neutral lipids. Phospholipids were removed by washing three times with methanol (Dittmer and Wells, 1969; see also for general reference). Acyl ester groups in all fractions were determined by the method of Rapport and Alonzo, (1955) using a tripalmitin (Sigma) standard. Total phosphorus was determined after a wet digestion with H_2SO_4 and H_2O_2 by the Fiske and Subbarow Method for P (Leloir and Cardini, 1957).

H.) Glutamate. Glutamate was determined with the Copper Salt micromethod for amino acids (Spies, 1957) with glutamate (Nutritional Biochemicals) as a standard.

V. Sources of Other Chemicals Used.

All inorganic chemicals used in this work were of reagent or comparable grade.

Peptone, yeast extract, Cantino PYG agar, and Casamino Acids (vitamin-free) were obtained from Difco Laboratories; Orcinol, EGTA, ADP, NADH, and D-mannitol from Sigma Chemical Company; Sucrose and Biebrich Scarlet from Matheson, Coleman and Bell; D-glucose from Eastman Organic Chemicals; EDTA from Fischer Scientific Company; Tris from General Biochemicals; α-ketoglutarate from Nutritional Biochemicals Corporation.

I. Encystment and Oxygen Uptake Studies

Because one of the objectives of this work was to study the physiology of zoospores of Blastocladiella emersonii before and during encystment, it was necessary to develop systems in which populations of these cells could either be maintained in the motile nonencysted state or be induced to encyst synchronously. Several systems have already been described by others, (Truesdell and Cantino, 1971; Soll et al., 1969), some after this work had been started.

In much of the preliminary work reported in this section, encystment and oxygen consumption were measured on the same system simultaneously. Therefore, the results are presented together here to facilitate understanding what happens to both these activities under various conditions, and to avoid repeating data from the same experiments in different sections.

The experimental procedure was similar to that developed by Cantino et al.,(1968; 1969). A summary of the manipulations and abbreviations is given in Table 1.

Three major S systems evolved from this work: (i) a sodium phosphate-calcium chloride system which was the first one used; (ii) modifications of the above to give high and low encystment values; and (iii) a MOPS-calcium chloride system which was finally used to maintain nonencysted populations.

A.) Sodium Phosphate-Calcium Chloride Salt Solution. In this system, S = 0.5 mM Na phosphate, pH 7.8, + 5 mM $CaCl_2$, W = 1, and T' = 0.5. In the following results, all of the other parameters

TABLE 1. Sequence for Preparation of Zoospores.

a.) OC plants grown on Petri plates of PYG agar at a density of 1-3 x 10^5 cells/plate.

b.) A salt solution (S) used to flood plates and obtain a suspension of zoospores after discharge of plants.

c.) Spores suspension in S filtered through Whatman #4 or Sargeant #500 paper.

d.) Spores either not washed (W=0) or washed (W=1) by two centrifugations at 1000 x g for 3 minutes. Spores resuspended in S.

e.) Spore suspension chilled for a time (T, in hours).

f.) Population density (P, in spores/ml) of spore suspension determined with the Coulter counter and adjusted with S.

g.) Spore suspension put in appropriate vessel for incubation at a defined temperature (C) for a time (T', in hours).

h.) Percent nonencystment (%NE) determined after T'.

(T, C, P, and %NE) were variables and are given for each experiment.

1.) Effect of Variable Incubation Temperature (C). Using a 0.5 hour chill period in this system, the amount of encystment depended on population density (Figure 2). This relationship was roughly logarithmic between 2×10^6 and 1.5×10^7 spores/ml and did not seem to be dependent on incubation temperature, since the points for 10 and 18 C fell in line with those for 22 C.

The oxygen consumption of these populations, consisting of both encysted and nonencysted spores is given in Table 2 under T = 0.5. As the %NE increased, (i.e. % encystment decreased) the Q_{O_2} (cell) decreased. These results, though not definitive, do indicate that a higher Q_{O_2} (cell) is associated with encystment. Factors which can cause variance in this system are mentioned in Results I, B.

The rate of oxygen consumption by these types of populations varied directly with temperature between 4 and 22 C (Figure 3) in populations with 60-90%NE. Although it was difficult to find the point at which oxygen uptake fell to zero, there was no detectable response at 4 C by the oxygen electrode for 30 minutes, while one was obtained in this time at 6 C.

2.) Effect of Variable Chill Period (T). The length of time a spore suspension is chilled before incubation affects percent encystment (Cantino et al., 1968; Truesdell and Cantino, 1971). This effect was also evident in Table 2. With no chill (T = 0), the populations consistently showed little encystment (high %NE) and a correspondingly low Q_{O_2} (cell). When T = 0.5 (Section 1, above) %NE was logarithmically related to P and was related also to a correspondingly variable Q_{O_2} (cell). With T = 1, however, %NE was uniformly

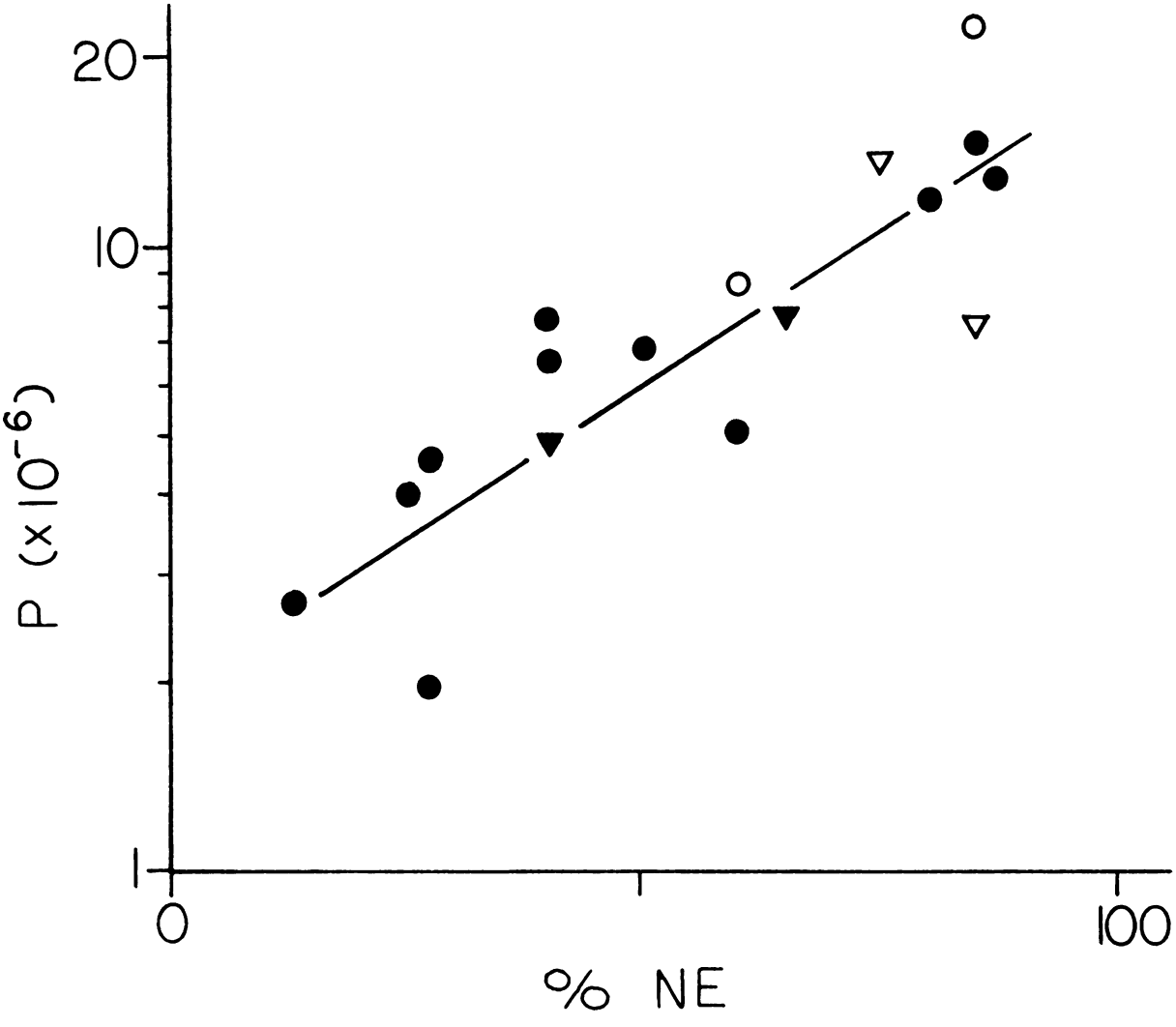

Figure 2. The Relationship Between Encystment and Population Density in the Phosphate-Calcium System. Symbols: 22 C, ● ; 18 C, ▼ ; 14 C, ▽ ; 10 C, ○ .

TABLE 2. The Effect of Chill Period and Population Density on Oxygen Uptake and Nonencystment.

T	P (x 10^{-6})	Q_{O_2} (cell)	%NE
0	2.3	12.6	75
	3.4	13.0	75
	5.2	11.6	75
	7.2	9.2	75
	9.4	9.8	70
	14.0	8.2	100
0.5	2.0	20.6	29
	4.0	17.8	25
	4.6	18.7	27
	6.5	16.4	40
	7.7	12.6	40
	11.7	11.3	80
	12.6	14.6	87
	14.3	14.6	85
1.0	4.2	15.8	25
	5.2	13.9	23
	8.5	12.9	23
	10.0	11.1	45
	13.0	12.1	60

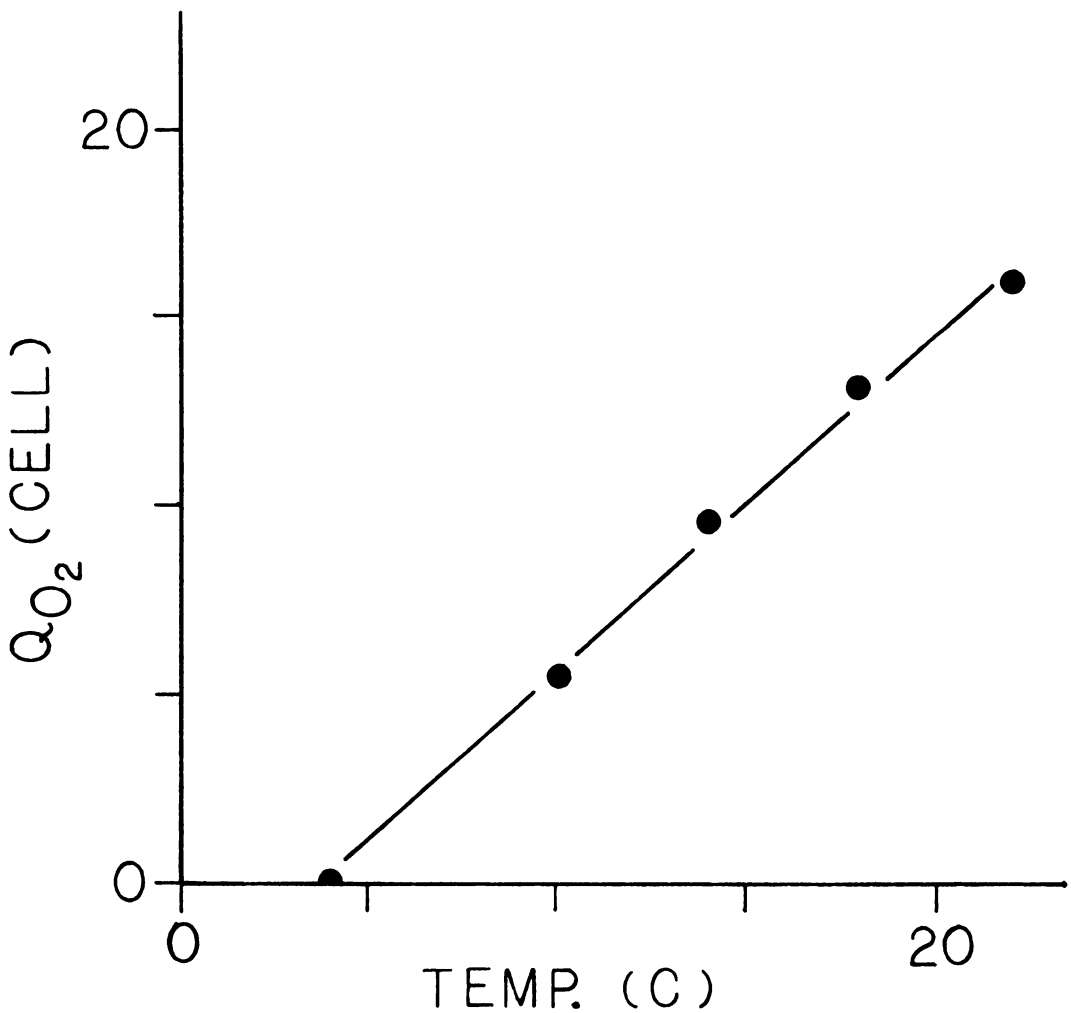

Figure 3. The Relationship Between Temperature and Oxygen Uptake. Points are averages of two determinations, except at 22 C, where the average is 8.

low, at least until P exceeded 10^7 spores/ml. The Q_{O_2} (cell) values here were not as high as for corresponding values of %NE when T = 0.5. The reason for this is not known, but low temperatures do affect mitochondrial shape (Cantino et al., 1969), and different salt solutions provide differing degrees of protection to the cell (Truesdell and Cantino, 1971). Therefore a one hour chill period in this system might be damaging to the respiratory activity of the cell.

3.) Effect of CO_2 on Oxygen Consumption. In order to determine the comparative effect of CO_2 on oxygen uptake in manometric and oxygen electrode runs, experiments with the Warburg were set up so that one set of spores respired in the absence of CO_2 (KOH in the center well), the other in the presence of CO_2 (Pardee's CO_2 buffer in the center well providing a constant level of CO_2 between 2-4% in the air phase, Umbreit et al., 1964). The spore suspensions, with T = 0.5 and C = 22, were monitored for oxygen uptake for two hours, and then checked for %NE. The results of four determinations with similar P (1.2×10^7 - 1.4×10^7 spores/ml) and %NE (70-90) are given in Table 3. Spores incubated in the presence of CO_2 had a slightly lower Q_{O_2} (cell) (7.8) than those in the absence of CO_2 (9.2).

TABLE 3. The Effect of CO_2 on Oxygen Uptake.

CO_2 in gas phase	Q_{O_2} (cell)				Average
present	8.0	8.3	6.7	8.4	7.8
absent	9.2	8.9	11.1	7.7	9.2

B.) Modifications of S to Induce or Prevent Encystment. In this section, the salt solution (S) and the length of the chill period (T) were varied in attempts to promote a large percentage of the population to encyst synchronously or to prevent the population from encysting.

1.) Biebrich Scarlet Induced Encystment. It had been shown (Truesdell and Cantino, 1971) that this sulfonated azo dye causes synchronous encystment of a large proportion of a chilled suspension of zoospores. A system with S = 5 mM Na phosphate, pH 6.8, W = 1, and T = 0.5 - 1.0 was used. Ten minutes before the end of the chill period, Biebrich Scarlet was added (final concentration, 0.1 mM). The spore suspension was then added to an oxygen electrode chamber at 22 C and oxygen uptake and encystment followed simultaneously. The Q_{O_2} (cell) rose in close correlation with the disappearance of nonencysted cells from the medium (Figure 4). The Q_{O_2} (cell) reached a maximum (21.8) at 14-16 minutes, as the %NE approached a minimum (2-5%), then decreased slightly. In almost all of these experiments and in experiments with other procedures, this slight decrease was noticed. This may have reflected a rapid depletion of some internal reserve at encystment (see Discussion) for respiration here was completely endogenous.

To further document the change in rate of oxygen uptake at encystment, experiments were done in which two batches of spores from the same population were used. One was not chilled, but was washed and suspended in 5 mM Na phosphate, pH 6.8, (B, in Figure 5). These spores did not encyst (%NE = 95) and showed a constant rate of oxygen consumption (Q_{O_2} (cell) = 8.0) for 30 minutes. The other batch was chilled for 1 hour in 5 mM Na phosphate, pH 6.8, Biebrich Scarlet added (final concentration was 0.1 mM), and incubated at 22 C (A, in Figure 5).

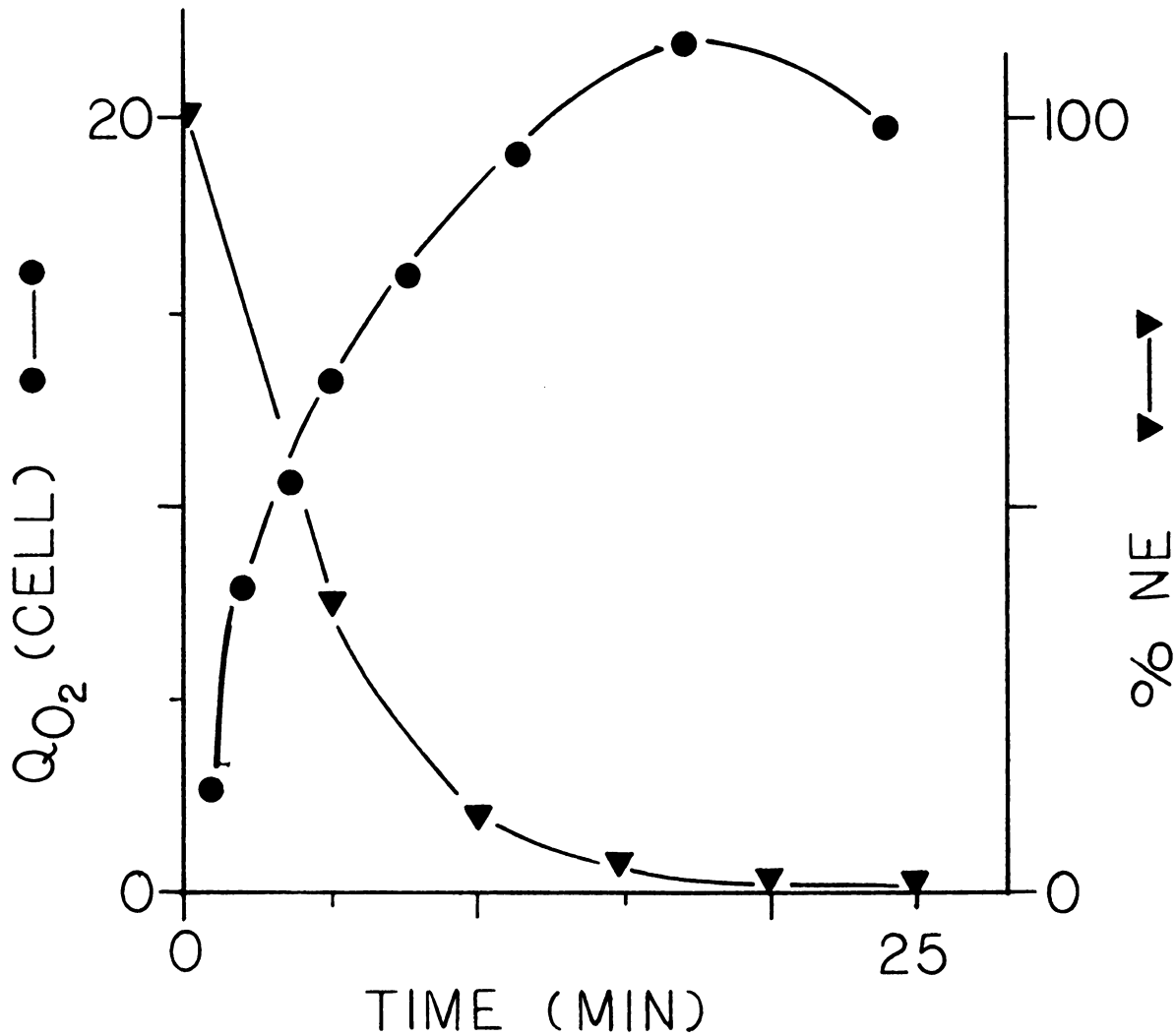

Figure 4. Kinetics of Encystment and Oxygen Uptake after Biebrich Scarlet Treatment. (%NE = 100-%E).

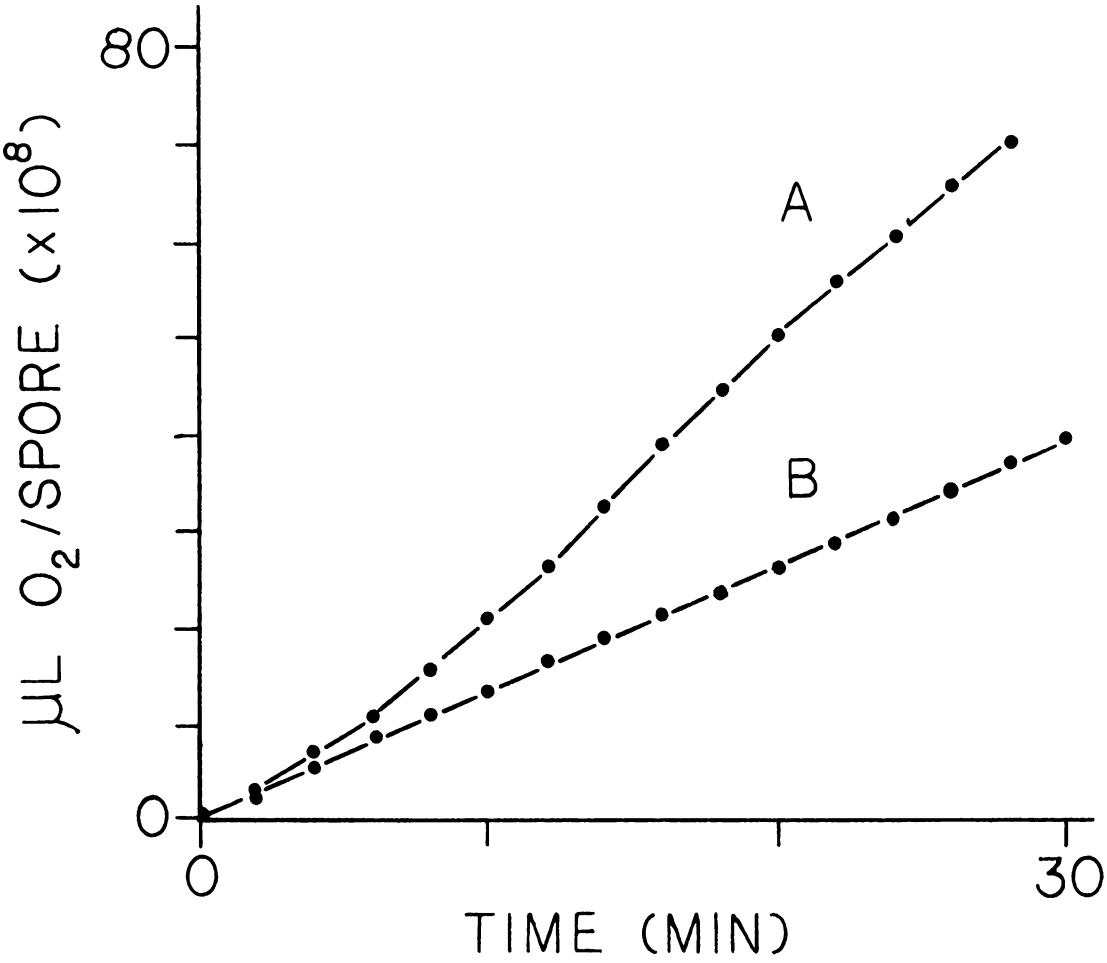

Figure 5. Oxygen Uptake of Encysted and Nonencysted Spores.
Curve A: spores induced with Biebrich Scarlet to encyst.
Curve B: nonencysted spores.

The encystment kinetics of this population were similar to that of Figure 4 with only 3-6%NE after 15 minutes. In contrast to the constant rate of oxygen uptake in Curve B, Curve A showed an increasing slope after the first four minutes which reached its maximum at ca. 15 minutes and then decreased slightly. The maximum Q_{O_2} (cell) reached was 19.4.

2.) Effect of Chill Period and Different Salts. The systems used in these experiments were essentially modifications of the system in Section I, A. The first was used to maintain a high %NE; in it, S = 0.5 mM Na phosphate, pH 7.0, + 1 mM $CaCl_2$ + 5 mM NaCl, T = 0, W =1, and C = 22. The results obtained (Table 4) were very similar to the results obtained with the original salt solution (Section I, A) with no chill period (see Table 2 for comparison). Here, however, the %NE seems to be related to P, although not enough data were collected for the lower population densities to fully uncover this relationship. Once again, there was an inverse relationship between Q_{O_2} and NE; at 95%NE the Q_{O_2} (cell) was 8.2.

TABLE 4. The Effect of Chill Period, Salts, and Population Densities on Nonencystment and Oxygen Uptake.

S	T	P (x 10^{-6})	Q_{O_2} (cell)	%NE
0.5 mM Na phosphate,	0	7.0	16.1	60
1.0 mM $CaCl_2$,		8.9	12.3	70
5.0 mM NaCl,		12.8	14.6	80
pH 7.0		13.7	10.3	90
		14.6	8.2	95
0.5 mM Na phosphate,	1	6.7	23.4	10
5.0 mM $MgCl_2$,		7.9	19.7	17
1.0 mM KCl,		9.3	20.9	5
pH 7.0		10.9	18.6	5
		12.4	16.5	34

Since Mg and K help induce encystment better than Ca and Na, (Cantino et al., 1968) the second modification made in an attempt to induce high levels of encystment involved a system consisting of S = 0.5 mM Na phosphate, pH 7.0, + 5 mM $MgCl_2$ + 1 mM KCl, T = 1, W = 1, and C = 22. The results of these experiments also given in Table 4, who that there was little relationship between P and %NE with such a long chill period (compare with Table 2, T = 1). This modification of S increased the percent encystment obtained with a 1 hour chill period and also gave higher Q_{O_2} values for the encysted population (19-21 for 5%NE) than the system in Results I, A, indicating that it perhaps provided more protection from the cold shock while still allowing encystment.

In a representative kinetic study using the Mg-K system, (Figure 6), the results were similar to corresponding experiments with Biebrich Scarlet (Figure 4), i.e. maximum Q_{O_2} and approaching a minimum %NE at ca. 15 minutes followed by a slight decrease in Q_{O_2}.

Other physiological activities also followed during the course of the type of experiment depicted in Figure 6, were levels of lactic acid in the medium and polysaccharide in the cell. No detectable lactate was found in the medium for two hours after encystment began, and the pH did not shift significantly. The internal content of a glycogen-like polysaccharide (BP) (Cantino and Goldstein, 1961) decreased dramatically more than 90% after 20 minutes. The kinetics of this loss and the corresponding %NE values are given in Figure 7. After a short lag, the content of polysaccharide decreased sharply, closely paralleling the encystment curve. Experiments with spores from plates and liquid cultures gave essentially the same results.

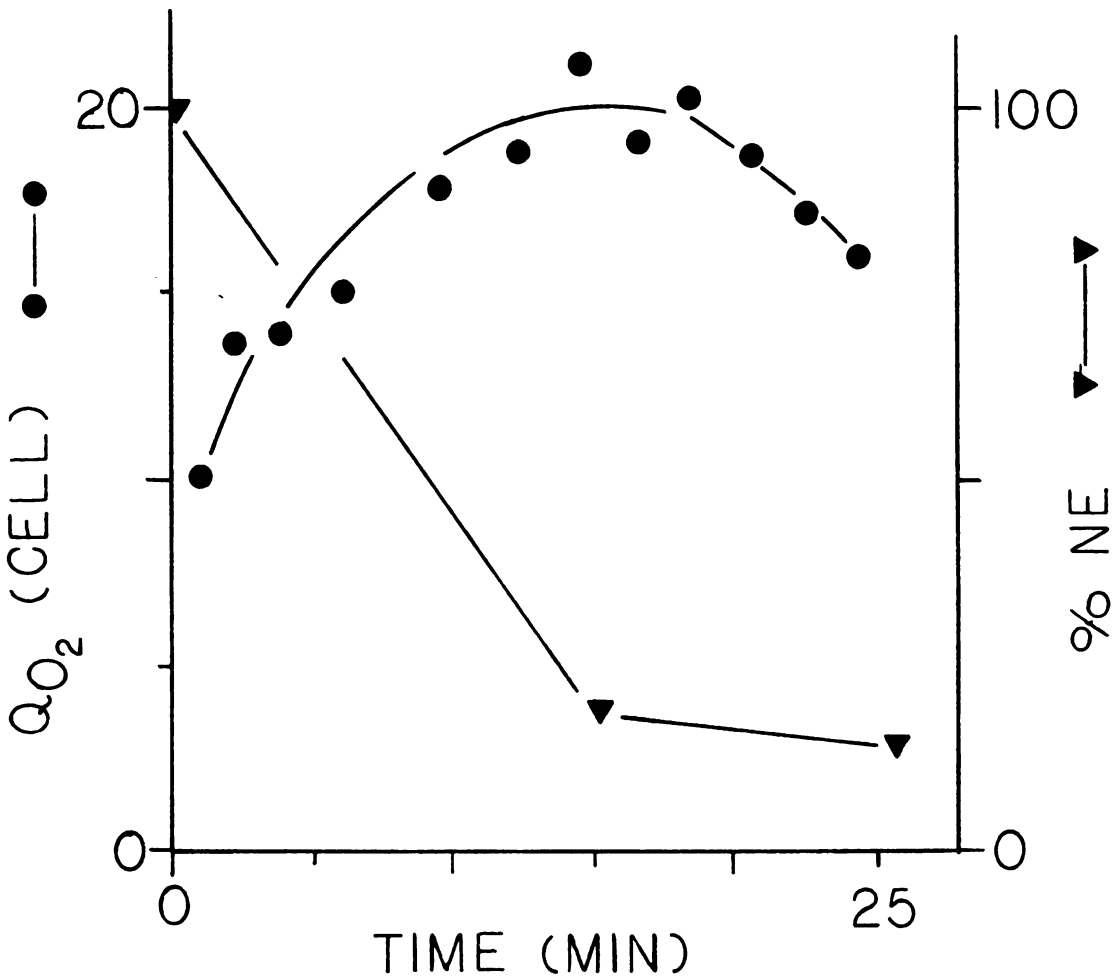

Figure 6. Kinetics of Encystment and Oxygen Uptake After Cold Treatment. (%NE = 100-%E).

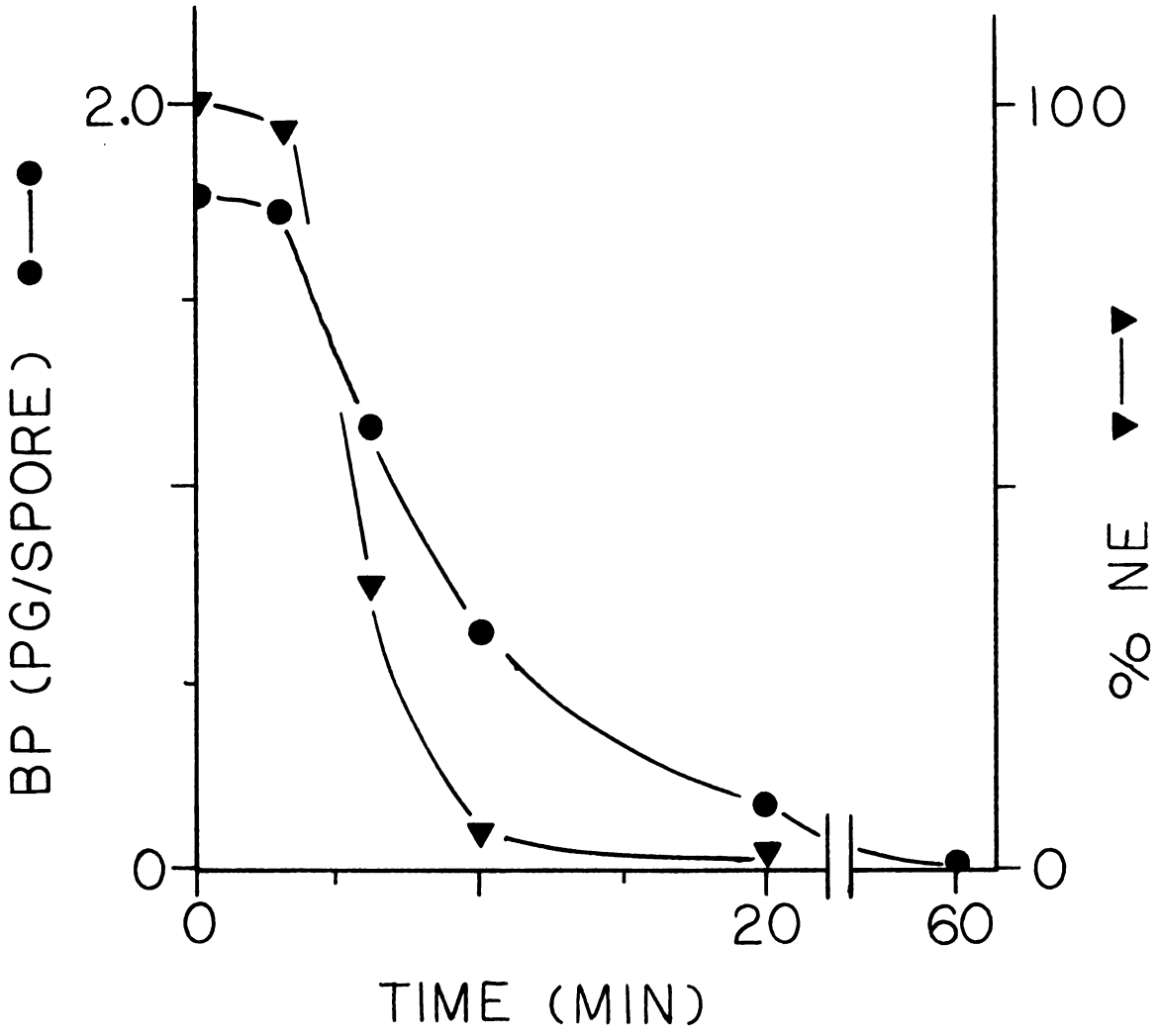

Figure 7. Kinetics of Encystment and Polysaccharide Loss After Cold Treatment. (%NE = 100-%E).

C. MOPS-Calcium Chloride Salt Solution. Since most of the above salt solutions had such a low concentration of phosphate buffer to prevent complexing and precipitation of calcium, and calcium had been shown to help prevent encystment, a new synthetic buffer, (see Good et al., 1966, for general reference) morpholino propane sulfonic acid (MOPS) was used. This buffer had the advantages of not combining with other ions in the medium, and had a pK_a of 7.2 (Good, personal communication) which is close to the pH values at which these studies were done.

1.) Effect of MOPS and Chill Period. To test the effect of this buffer on zoospores of B. emersonii, studies of encystment were done with S = 5 mM MOPS, T = 0.5, W = 1, C = 22, and T' = 0.5. Three pH values were used: 6.7, 7.2, and 7.8. The results (Figure 8) show that at all three pH values, %NE was a logarithmic function of P, in the same manner as in Figure 2. The intercept increased slightly with pH, but the slope remained constant.

2.) Effect of Calcium and Chill Period. With the addition of 1 mM or 10 mM $CaCl_2$ to the MOPS system, the relationship between P and NE was markedly altered (Figure 9) with the 0.5 hour chill period, %NE is high (80-100) and constant at population densities up to ca. 6×10^6 spore/ml. Above this, however, more cells encyst with increasing P, just as the reverse of the effect obtained in this system without $CaCl_2$, and in the sodium phosphate-calcium chloride system of Results I, A. Perhaps this indicates calcium-phosphate complexes were being formed in the first system.

3.) Effect of Growth Temperature of OC Plants on Encystment. Three growth temperature ranges were used: 18, 23-24, and 27-29 C. With S = 5 mM MOPS, pH 6.8, + 10 mM $CaCl_2$, W = 1, T = 0.5, C = 22, and

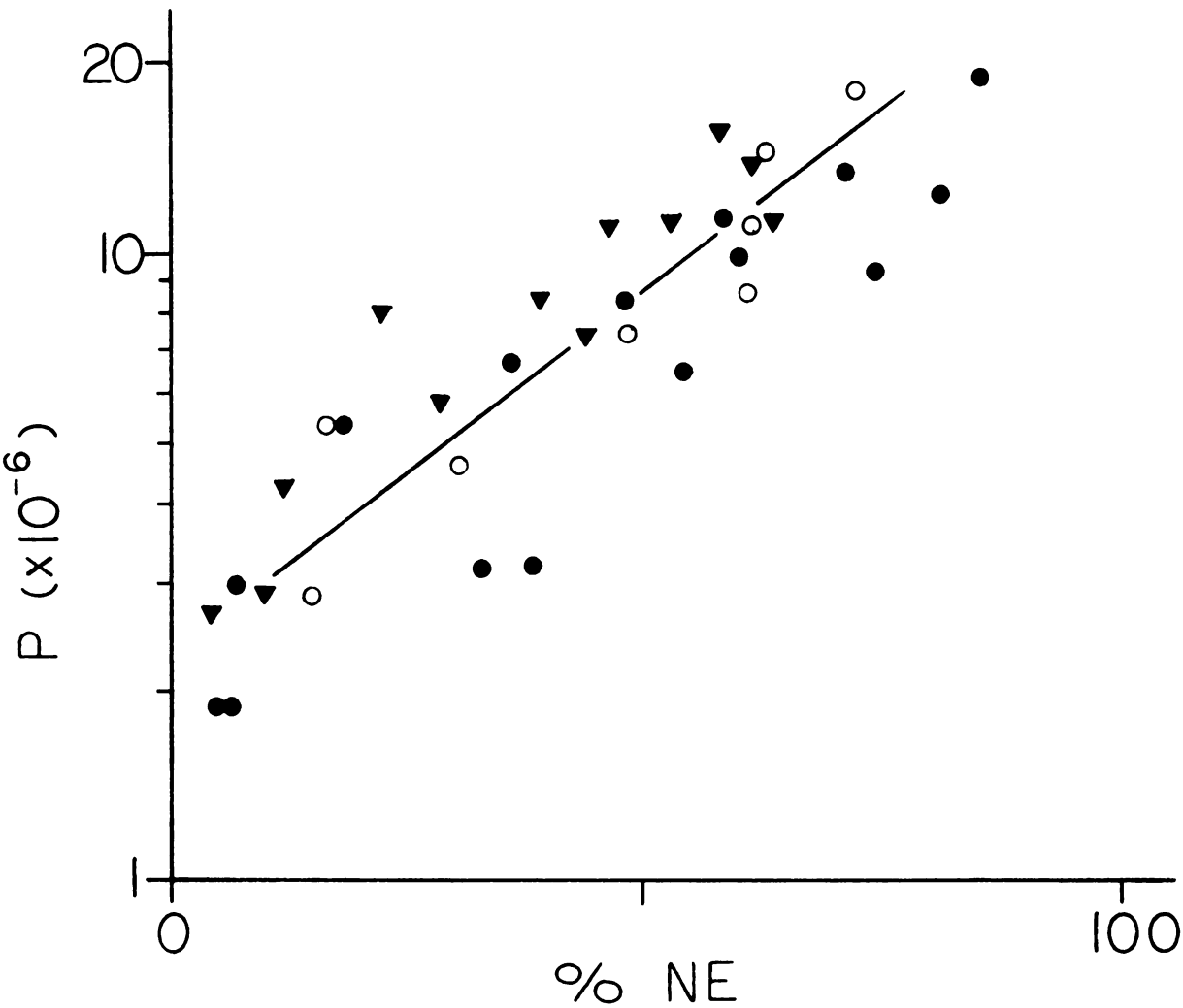

Figure 8. The Relationship Between Encystment and Population Density in the MOPS System. pH symbols: 6.8, ● ; 7.2, O ; 7.8, ▼ .

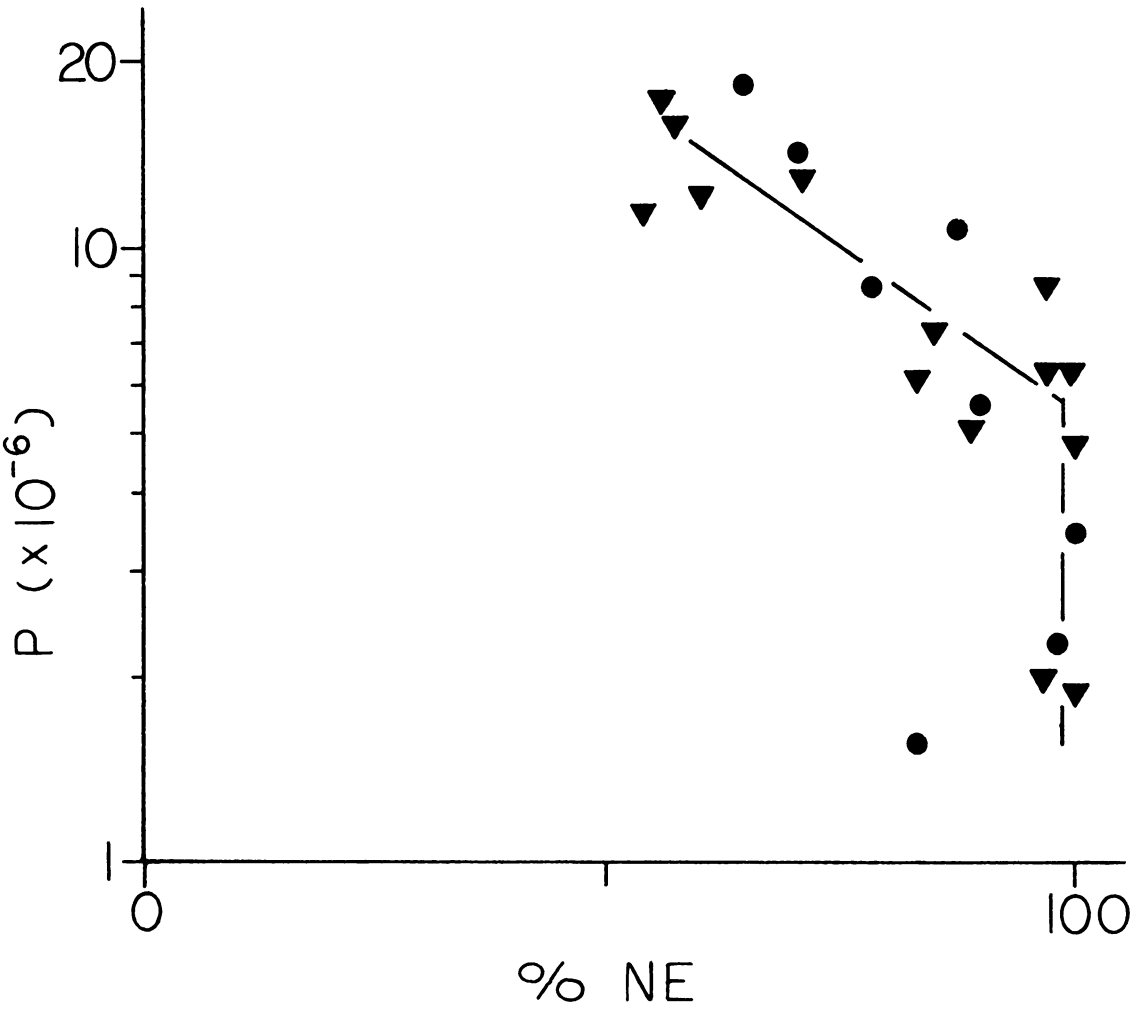

Figure 9. The Relationship Between Encystment and Population Density in the MOPS-Calcium System. Symbols for calcium concentration: 10 mM, ▼; 1 mM, ● .

T' = 0.5, the lower the temperature the more cells remained nonencysted (Figure 10). The chill period was important (see Discussion) since without it zoospores did not encyst (see following section).

4.) Effect of No Chill Period. With T = 0, and S = 5 mM MOPS, 5 mM MOPS + 1 mM $CaCl_2$, or 5 mM MOPS + 10 mM $CaCl_2$, %NE is consistantly high (90-100) at all population densities (Table 5). Because of the lack of encystment in these systems, this type of system was used for all further physiological experiments on zoospores. Data for oxygen consumption and other physiological changes are presented in Results III.

TABLE 5. The Effect of the Absence of a Chill Period on Nonencystment in MOPS and MOPS-calcium Systems.

5 mM MOPS,	pH 6.8	5 mM MOPS, 1 mM $CaCl_2$	pH 6.8	5 mM MOPS, 10 mM $CaCl_2$	pH 6.8
P($\times 10^{-6}$)	%NE	P($\times 10^{-6}$)	%NE	P($\times 10^{-6}$)	%NE
14.3	96	15.0	98	12.2	98
11.8	98	14.9	94	7.6	100
9.6	98	10.6	94	4.4	98
7.9	93	9.3	98	1.6	98
6.8	94	7.4	88		
4.7	93	5.0	91		
2.0	92	2.3	94		
1.6	94	1.3	96		

5.) Effect of Deflagellation on Oxygen Consumption. The oxygen consumption of zoospores incubated in MOPS-calcium solutions with T = 0 were either used directly without treatment or deflagellated by either of two methods: (i) spun on a vortex mixer for 20 seconds (Soll and Sonneborn, 1969), or (ii) aerated vigorously for three minutes (Cantino et al., 1968). The degree of deflagellation and %NE was later determined on fixed samples of the respective spore suspensions

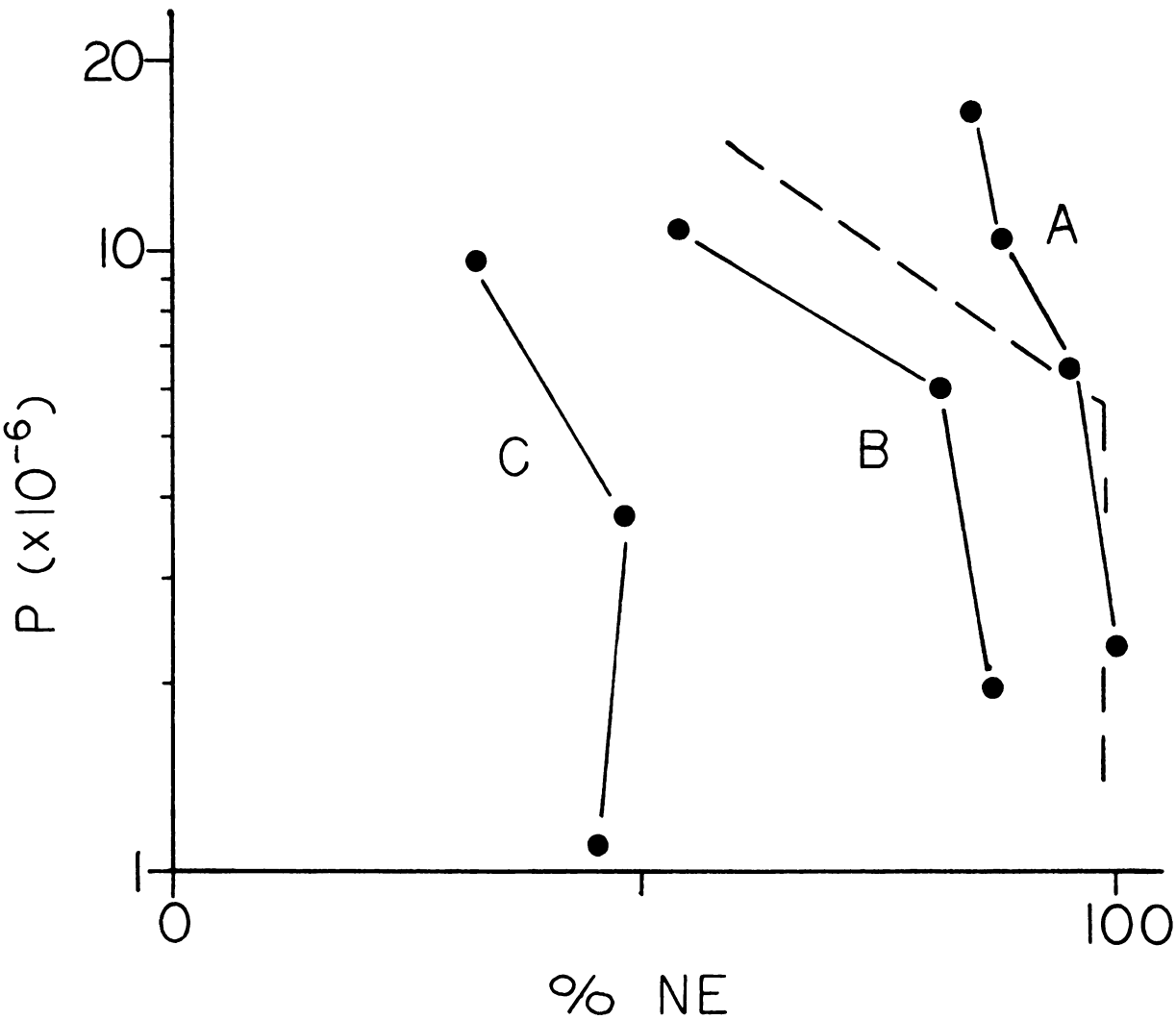

Figure 10. Effect of Growth Temperature of Parent OC Plants on Encystment. Curve A: 18 C. Curve B: 23-24 C. Curve C: 27-29 C. The dashed curve is for 22 C and is from Figure 9.

and the results are given in Table 6 along with the results for oxygen consumption. Although the difference between the flagellated and deflagellated with vortex mixer should be considered a minimum value since only the outer portion of the flagellum was removed and the values for flagellation were not 100% and 0%, there was a drop of 15% in the Q_{O_2} of the spores without flagella (see Discussion).

TABLE 6. The Effect of Deflagellation on Oxygen Uptake.*

Condition of Spores	Q_{O_2} (cell)	% flagellated	%NE
flagellated	9.0	90	93
deflagellated with aeration	8.3	18	82
deflagellated with vortex mixer	7.7	7	96

* Each value is the average of three determinations. S = 1 mM MOPS, pH 6.8, +1 mM $CaCl_2$, T = 0, W = 1, and C = 22.

II. Liquid Cultures.

To obtain enough zoospores for physiological studies, various modifications of liquid culture methods were examined to determine which ones gave maximum yields of zoospores with a high degree of synchrony. Liquid cultures were preferred over plate cultures because: (i) they provided a homogenous environment for growth; (ii) they were easier to monitor for the stages of development of the population; and (iii) they required fewer manipulations in inoculation and harvesting for the yields obtained.

Several modifications of the basal PYG liquid medium have been described (See Lovett, 1967, for review). In this work, PYG-P and

PYG-PC were used (see Materials and Methods). Other variables tested were either no induction or induction with one of three solutions (see Materials and Methods), and the effect of light. Because B. emersonii grows more rapidly in the light (Cantino, 1965), and because light plays a role in nuclear divisions, different light regimes were used in an attempt to improve synchrony and yield.

A.) Noninduced Cultures. Good growth of the plants was observed in cultures allowed to grow and develop without induction, whether in PYG-P, PYG-PC, or modifications of these (Table 7), but there was very little spontaneous differentiation into zoosporangia as occurs in Blastocladiella grown on plates of the same media. It mattered very little whether these cultures were grown in total darkness or in continuous light. Many of the plants were allowed to grow for up to 42 hours and upon examination gave the impression of developing along the RS pathway. The plants had continued to grow, formed a septum, and superficially looked like the 30-36 hour RS plants of Lovett and Cantino (1960). These plants were not allowed to mature, hence it was not established whether or not they were true resistant sporangial plants; they may have been the "pseudo RS" types described by Domnas (1968).

B.) Induced Cultures. Using the induction procedure described in Materials and Methods, plants grown in both media were made to differentiate and discharge. Table 8 shows results with cultures induced after 17-18 hours of growth with each of the three induction solutions. The results are tabulated in terms of yield, i.e. total zoospores recovered from 1.2 liters of culture media, fold increase, i.e. number of zoospores produced divided by the number in the inoculum, and per cent

TABLE 7. Representative Noninduced Liquid Cultures.*

Culture number	Medium	Inoculum (spores/ml)	Light conditions	Results
1	PYG-P(K^+)	1.2×10^4	continuous light	In all cases, there was good growth of the plants, but only ca. 1% papilla formation and discharge after 24 hours. After 42 hours, most plants looked like developing ?S plants.
2	PYG-P(K^+)	1.4×10^4	continuous dark	
3	PYG-P(K^+)	3.0×10^4	continuous light	
4	PYG-P(equimolar K^+ and Na^+)	3.4×10^4	continuous light	
5	PYG-P(Na^+)	3.3×10^4	continuous light	
6	PYG-PC	1.0×10^4	continuous light	
7	PYG-PC	3.0×10^4	continuous light	

* Cultures were 1.2 liters, grown at 24 C, aerated with 6 liters air/min. Values represent 2-3 attempts.

TABLE 8. Representative Induced Liquid Cultures.*

Culture number	Medium	Inoculum (spores/ml)	Inducing solution	Total yield	Fold increase	% discharge per hour
1	PYG-PC	9.0×10^4	Calcium-citrate	3.7×10^8	3	16
2	PYG-PC	5.0×10^4	Modified $\frac{1}{2}$ DS	5.2×10^8	9	36
3	PYG-P(K^+)	1.2×10^4	Modified $\frac{1}{2}$ DS	1.3×10^9	91	66
4	PYG-P(K^+)	1.0×10^4	10x MOPS-calcium	very few discharged; papillae abnormal and long.		
5	PYG-P(K^+)	1.0×10^4	MOPS-calcium	1.5×10^9	125	79
6	PYG-P(K^+)	1.0×10^4 to 5.0×10^4	MOPS-calcium	4.1×10^9 to 1.9×10^{10}	84 to 91	68

* Cultures were grown at 22 C, with light regime of L-D-L (for details, see text). Cultures 1-5 are averages of 2-3 experiments; they were 1.2 liter cultures, aerated with 6 liters air/minute, and induced at 17 hours. Culture 6 is the average of 8 experiments which were used for the physiology experiments of section III. All were 4.5 liter cultures, and were aerated with 10 liters air/minute, and induced at 18 hours.

discharge/hour, i.e. average rate of discharge from that portion of the curve where the rate was fairly constant (see Figures 11-14). The light regime for cultures 1-4 was, successively, 6 hours light, 9 hours dark, and 9 hours light; for cultures 5-6, it was 11 hours light, $4\frac{1}{2}$ hours dark, and $8\frac{1}{2}$ hours light. The kinetics of papilla formation and discharge (the two morphological markers followed) are plotted in Figures 11-14.

Plants grown in PYG-PC developed fairly synchronously through papilla formation, but the discharge rate was low indicating that the culture had become less synchronous after papilla formation (Cultures 1-2, Table 8 and Figures 11-12). Culture 1 induced with calcium-citrate, had a lower discharge rate than culture 2 which was induced with modified $\frac{1}{2}$ DS (compare Figure 11 and 12).

Plants grown in PYG-P (cultures 3-6 and Figure 13-14) and induced with modified $\frac{1}{2}$ DS and MOPS-calcium were more synchronous in both papilla formation and discharge than those grown in PYG-PC. The MOPS-calcium solution gave the higher rate of discharge and hence better synchrony (compare Figure 13 and 14). However, a tenfold increase in MOPS-calcium (culture 4, Table 8) gave only about 50% papilla formation and very few discharging plants. Those papillae which did form were long and misshapen and looked abnormal. Since the standard MOPS-calcium system gave slightly better synchrony and maximum yields and since it had already been selected as the incubation medium for physiological studies of nonencysted spores, it was used in subsequent work. The results plotted in Figure 14 (culture 6, Table 8) are therefore representative of many cultures from which the spores were used in later experiments. The system finally perfected produced ca. 100% discharge

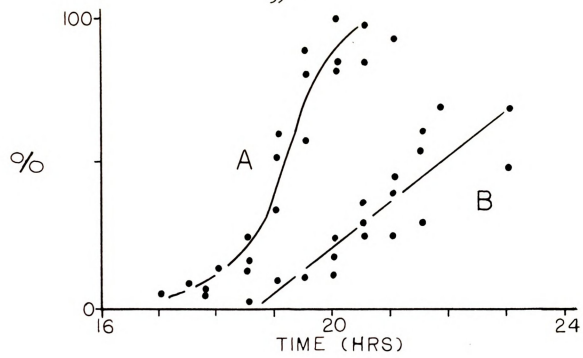

Figure 11. Synchrony in PYG-PC with Calcium-Citrate Induction. Curve A: % papilla formation. Curve B: % discharge. See Table 8, Number 1, for further details.

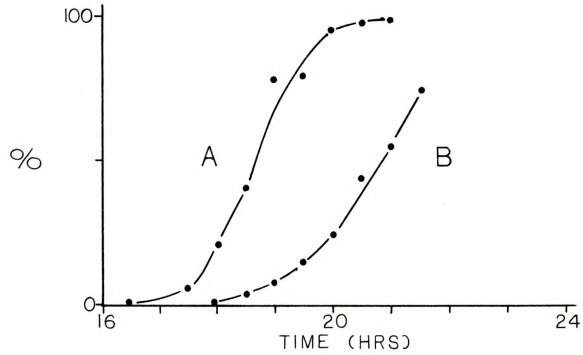

Figure 12. Synchrony in PYG-PC with $\frac{1}{2}$ DS Induction. Curve A: % papilla formation. Curve B: % discharge. See Table 8, Number 2, for further details.

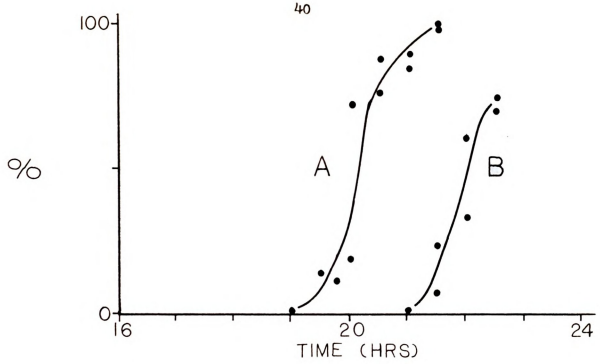

Figure 13. Synchrony in PYG-P with $\frac{1}{2}$ DS Induction. Curve A: % papilla formation. Curve B: % discharge. See Table 8, Number 3, for further details.

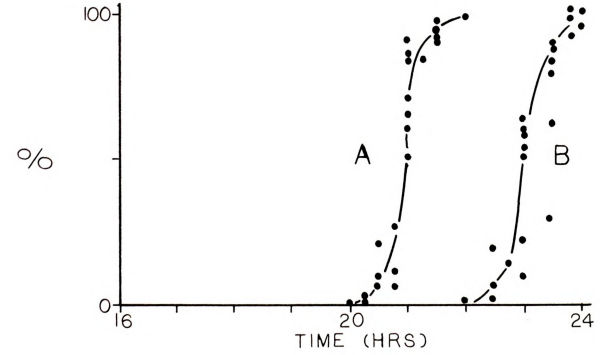

Figure 14. Synchrony in PYG-P with MOPS-Calcium Induction. Curve A: % papilla formation. Curve B: % discharge. See Table 8, Number 6, for further details.

in the final 1.5 hours of a 24 hour generation time. Typical stages of development of the plants after the cultures had been induced are shown in Figure 15. The plants were rather uniform in size, had generally one papilla and usually produced ca. 250 spores/plant.

Since all previously induced cultures had been exposed to light-dark-light regimes of about the same proportions, the effect of continuous light and continuous darkness was determined with cultures induced with the MOPS-calcium solution. There was essentially no difference in synchrony between the two (Figure 16) and the synchrony was approximately the same as for a light-dark-light regime with the same inducing solution (Figure 14).

III. Endogenous Physiology.

Using the liquid culture system finally developed in the preceding section to produce large numbers of zoospores synchronously, and the MOPS-calcium incubation system to keep them from encysting, endogenous physiological and biochemical activities were followed in zoospores. Table 9 provides zero time reference values, both in absolute terms and as percentages of dry weight, for the major chemical constituents of the cell. Protein made up the largest component of the cell, followed by nucleic acid, lipid and glycogen-like polysaccharide. These constituents accounted for over 75% of the dry weight, and all were followed during incubations of swimming zoospores to determine which were utilized during endogenous metabolism to provide the energy requirements for these cells.

A.) Per cent Nonencystment. In all experiments, samples were removed at intervals, fixed with glutaraldehyde and later counted in a

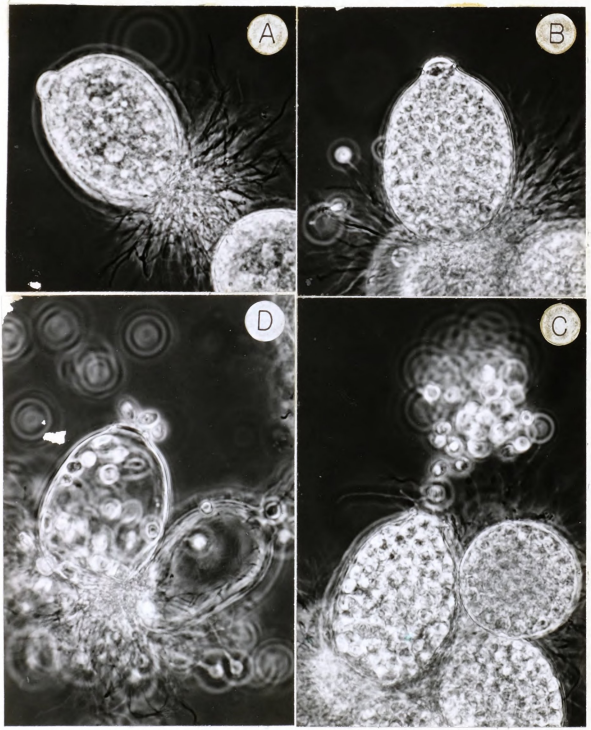

Figure 15. Papilla Formation and Discharge of OC Plants in Liquid Cultures. A. Early papilla formation. B. Late papilla formation (spores being cleaved). C. Beginning of discharge. D. Late discharge (sporangia almost empty). Plants are ca. 60 x 80 μ.

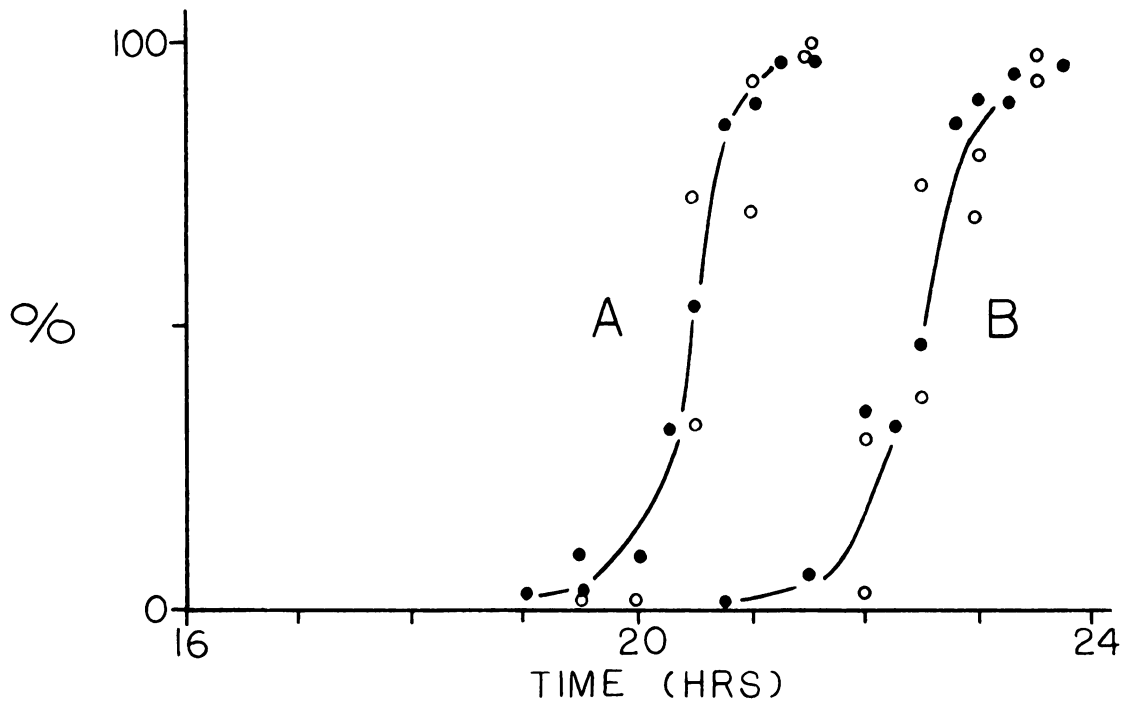

Figure 16. Effect of Light and Darkness on Synchrony in PYG-P. Cultures were 1.2 liters and induced at 18 hours with MOPS-Calcium. Curve A: % papilla formation. Curve B: % discharge. Open points represent cultures in continuous light; solid points, continuous darkness.

TABLE 9. Composition of the Zoospore of B. emersonii.

Component	pg/spore	% of Dry Weight
Dry weight	47.0	100
Nucleic acid	7.6-10.8	16-23
Protein	25.2-26.8	54-57
Total N	5.2-5.5	11-12
Total lipid	3.5-4.1	7-9
Phospholipid	3.2	7
Polysaccharide	1.4-2.3	3-5

TABLE 10. Encystment and Nonencystment in Endogenous Physiology Experiments.*

Time (hrs.)	%NE	%E
0	100	1.4
1	98	2.0
2	100	2.4
3	101	1.8
4	98	2.5
5	100	2.2

* %NE values are averages of 4 determinations; %E of 3.

haemocytometer to monitor the population for nonencysted spores and, in some cases, encysted spores. Table 10 shows the averages for the 5 hour period. The %NE remained close to 100% throughout. The data for encysted cells (%E) also support this, these values increased less than one percentage unit during this time. It is thought that the 1.4% encysted

cells at zero time represented the few cells which were disturbed enough during centrifugation to cause encystment. It should be noted that with the conditions of endogenous metabolism used, these cells were not viable, i.e. became dark under phase contrast after 1-2 hours and, therefore, probably contributed little to the changes observed for the total population.

B.) Oxygen Consumption. For studies using the oxygen electrode samples were removed at 0, 2 and 4 hours, put in the electrode chamber, and the constant rate of uptake over a 30 minute period determined. For measurements with the respirometer, samples were taken at zero time and O_2 consumption followed at 20 minute intervals for 5 hours. Uptake was linear over the entire 5 hour period. The uptake data are presented in Table 11. The average Q_{O_2} of zoospores, as measured with the oxygen electrode, decreased slightly during the 4.5 hour incubation from 9.4 to 7.8. This initial value is in good agreement with that in other systems (see Results, I, B), and also corresponds closely with the average Q_{O_2} of 9.8 determined manometrically for spores from the same culture. The slight difference (9.4 versus 9.8) could be related to the CO_2 levels (see Results I, A and Discussion), since Warburg vessels had KOH in the center wells and, therefore, contained very little CO_2.

The Respiratory Quotient (R.Q.) was also determined in the course of the Warburg incubations. Duplicate flasks with and without KOH in the center wells were used, HCl was tipped into the spore suspension incubated without KOH at the end of the run (Umbreit et al., 1964), and the production of CO_2 over the 5 hour period was determined. The average R.Q. was calculated to be 0.92.

TABLE 11. Oxygen Uptake in Endogenous Physiology Experiments.*

Method of determination	Time (hrs.)	Q_{O_2} (cell)
Oxygen electrode	0	9.4
	2	8.1
	4	7.8
Manometric	0-2	9.8
	2-4	9.9

* Oxygen electrode values are averages of 4 determinations each; manometric of 8.

C.) Dry Weight. The dry weight of zoospores at 0 and 5 hours was determined. The average of three determinations with two replicas each, was 47.0 pg/spore for zero, and 45.5 pg/spore for 5 hours, and the average loss was 1.5 pg/spore. Since the zoospores are so fragile and could not be collected and washed by filtration, the medium was also dried to constant weight and the values corrected for the MOPS-calcium solution (see Material and Methods IV, A). Therefore, this loss in dry weight should be considered a minimum value, because any nonvolatile metabolite released into the medium would still be considered part of the cell with this procedure.

D.) Nucleic Acid. The nucleic acid content of the zoospores remained constant over the incubation period (Figure 17). It has been impossible to demonstrate synthesis of RNA by zoospores before or even up to 30-40 minutes after encystment (Lovett, 1968). This fraction remained constant indicating that it was not broken down and utilized during endogenous metabolism. These data, along with the cell counts, also served to verify the maintenance of a constant

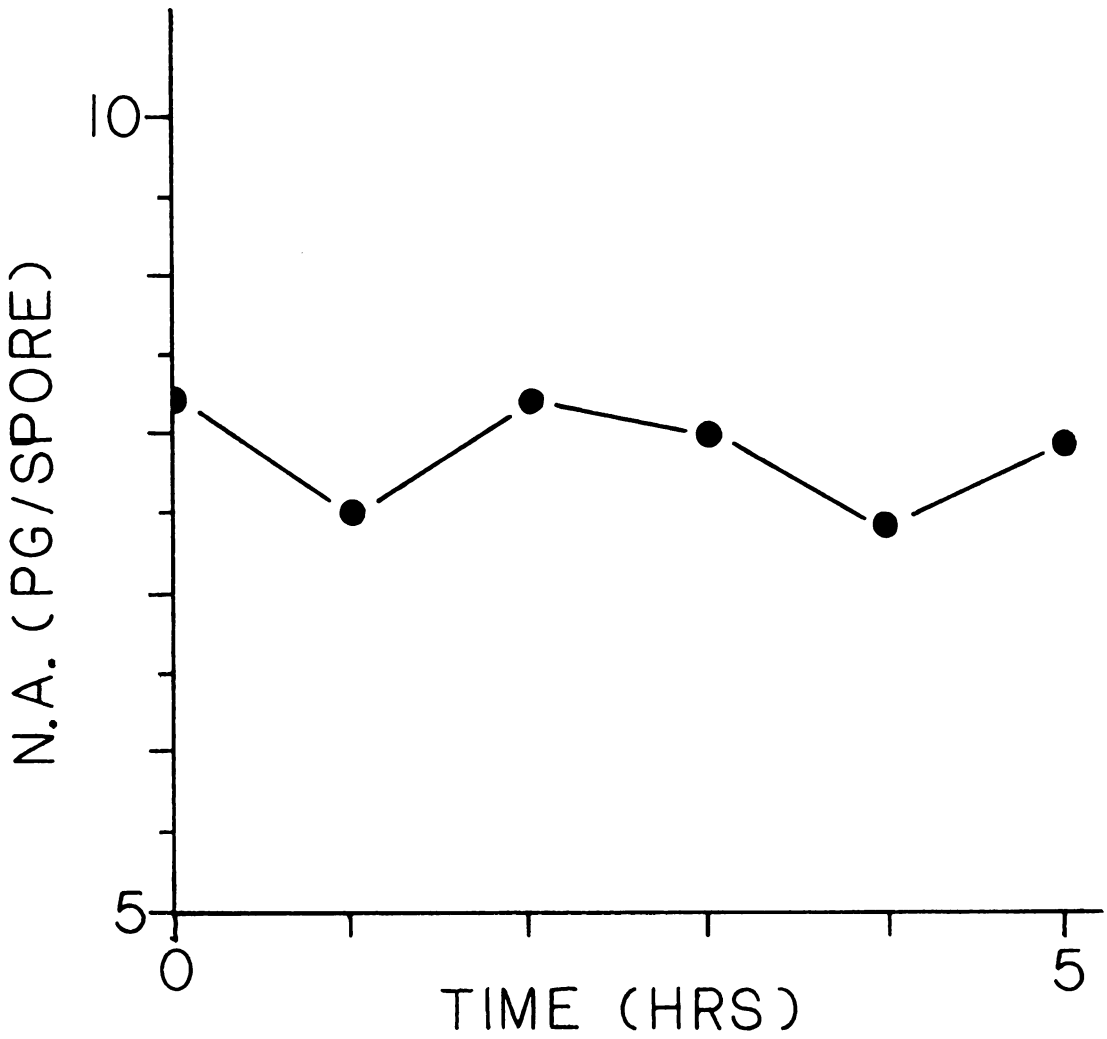

Figure 17. Level of Nucleic Acid During Endogenous Metabolism. Points are averages of 5 experiments.

cell number in the population. If there were cell breakage, this component would also have decreased.

E.) Protein. TCA precipitable protein decreased with time (Figure 18). It appears that protein did not disappear at a constant rate, but almost leveled off between 2 and 3 hours and then decreased at a lower rate. The hourly loss for the first two hours was 0.9 pg/spore, that for the last two was 0.5 pg/spore. The overall loss of protein in 5 hours was ca. 3.0 pg/spore or 11.5% of the protein.

The initial zero hour protein content did not vary much from experiment to experiment, i.e. from 25.2 to 26.8 pg/spore with an average value of 25.9 pg/spore. In all experiments, the average hourly losses were similar (0.6 pg/spore), no matter what the initial value was. This contrasts sharply with the data for polysaccharide and uptake of exogenous substrates (see Results III, G and IV).

Other indications that protein was being broken down by the cell come from data on total cell N and the accumulation of ammonium ions in the medium. Total N at 0 and 5 hours was measured in two experiments; it decreased from 5.4 to 5.0 pg/spore, i.e. with a loss of 0.4 pg N/spore in 5 hours. Correspondingly (Figure 19), NH_3-N accumulated in the medium with increasing rate. The last measurement made yielded a value of 0.38 pg NH_3-N/spore, almost identical to the amount of N lost. Along with this, a rise in pH of the medium was also observed (Figure 19). Assuming 16% N in protein, the loss of 0.4 pg N/spore and the corresponding gain of NH_3-N in the medium is equivalent to a calculated loss of 2.4 pg protein/spore, which is very close to the Lowry value of 3.0 pg protein/spore determined experimentally.

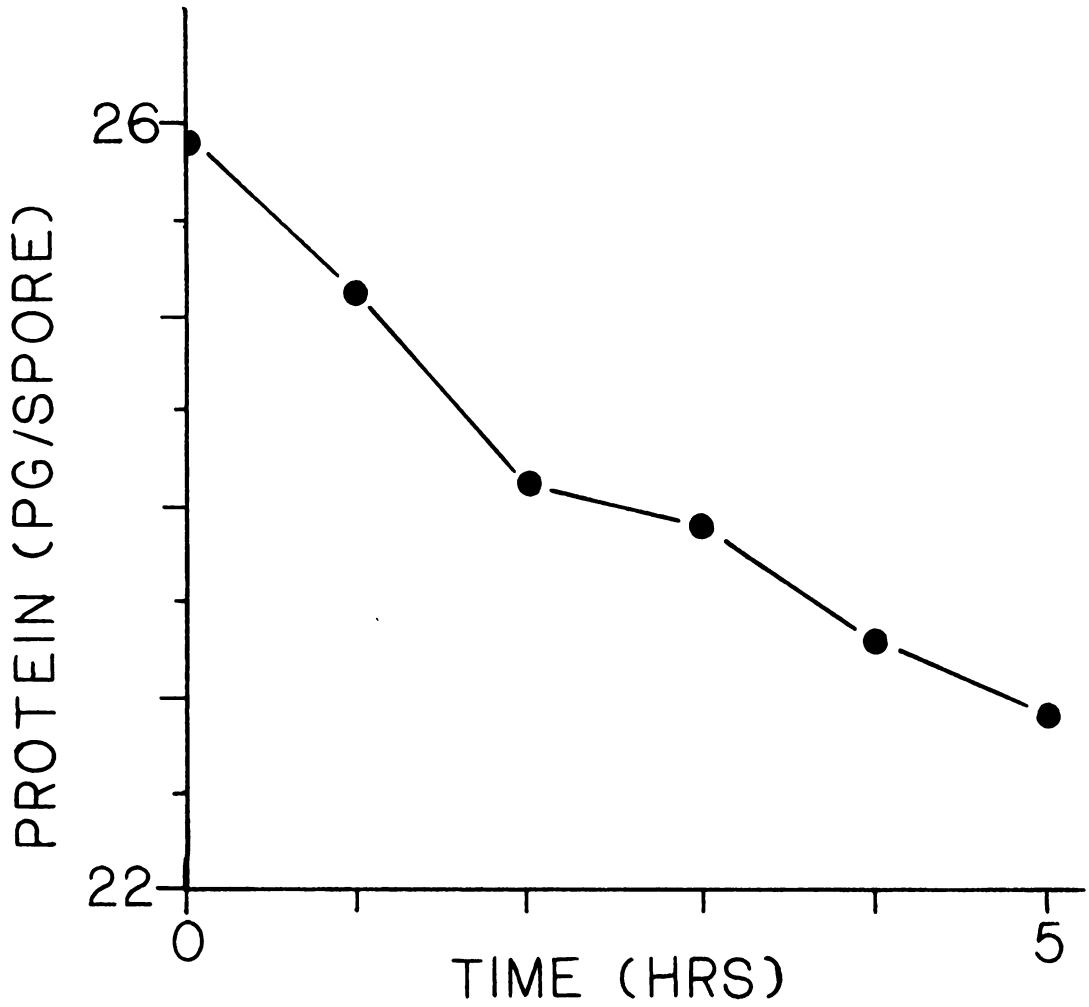

Figure 18. Protein Content During Endogenous Metabolism. Points are averages of 5 experiments.

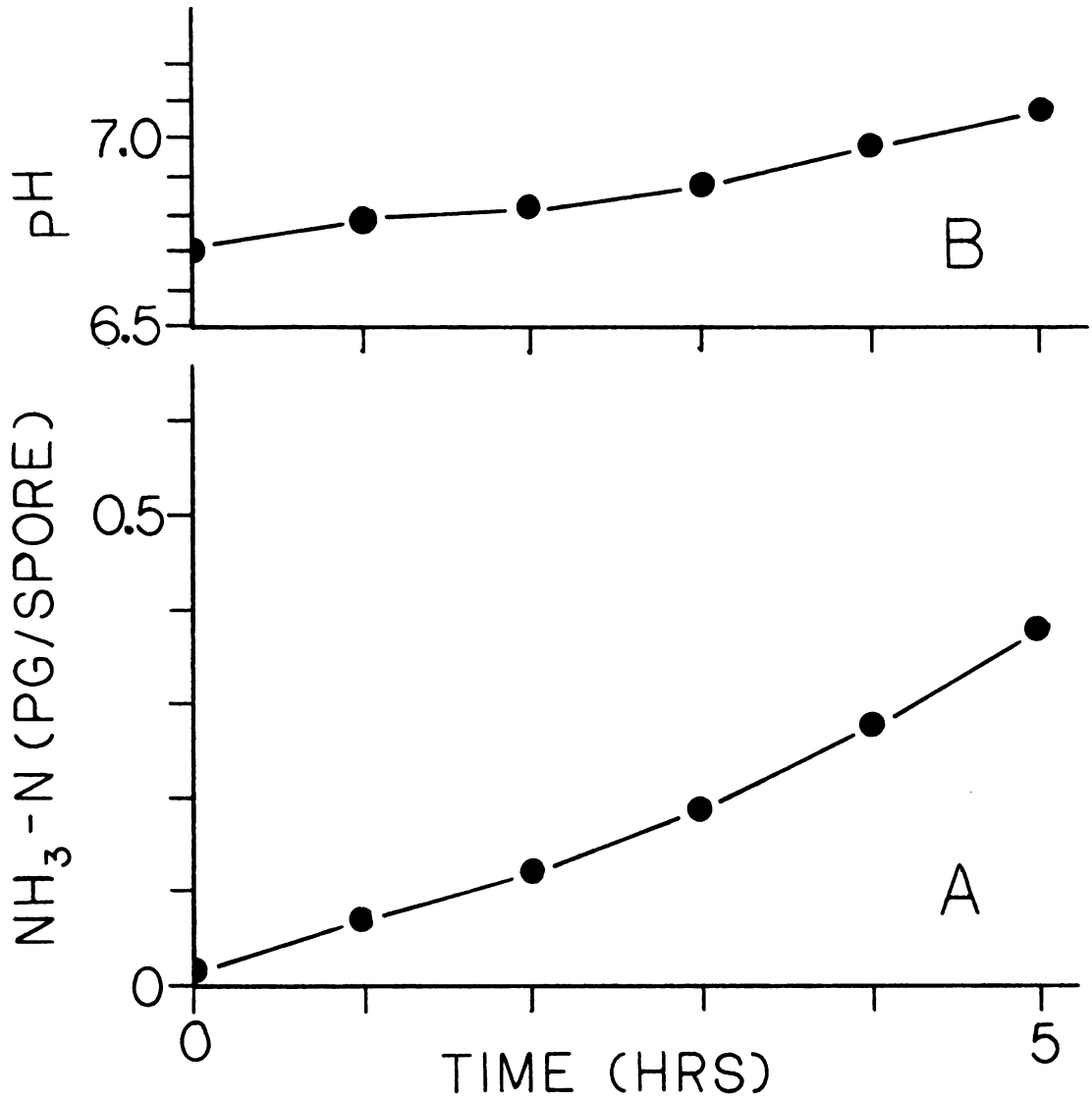

Figure 19. Changes in pH and NH_3-N in the Medium During Endogenous Metabolism. Points are averages of 2 experiments.

F.) Lipid. Lipid also decreased during endogenous metabolism (Figure 20). Using the dichromate method, the total loss was 0.95 pg/spore in 5 hours, approximately twice as much being lost in the first period as in the last. Using dry weight determinations, the total loss was 0.94 pg/spore, although the absolute values were higher (4.86 pg/spore for initial dry weight value vs. 3.87 pg/spore for initial dichromate value).

The total lipid was also separated into neutral lipid and phospholipid fractions with silicic acid (see Materials and Methods IV, G) and all three analyzed for fatty acyl esters and phosphorus (Table 12). The silicic acid separation was very good for phospholipid, as determined from the data for P contents of the total extract and phospholipid fraction which were almost identical. The quantity of fatty acyl ester groups in the phospholipid fraction decreased proportionately with phosphorus content, as seen in the relative constancy of the ratio μeq acyl ester/umoles P (1.6 out of a theoretical maximum of 2, Table 12). Also given in Table 12 are μeq acyl ester of the neutral fraction of the extract, calculated by taking the difference between μeq of acyl ester of the total extract and phospholipid fractions.

Absolute quantities of both phospholipid and triglyceride were approximated using conversion factors. To obtain μg phospholipid, the value for μg P was multiplied by 25 (see Lees, 1957). To obtain μg triglyceride, the value for μeq was multiplied by 288 (one third the average molecular weight, 863, of a triglyceride composed of three of the most common fatty acids, palmitic, stearic and oleic). Although these approximations are subject to some error, they should be representative enough for considerations here; they are plotted in Figure 21.

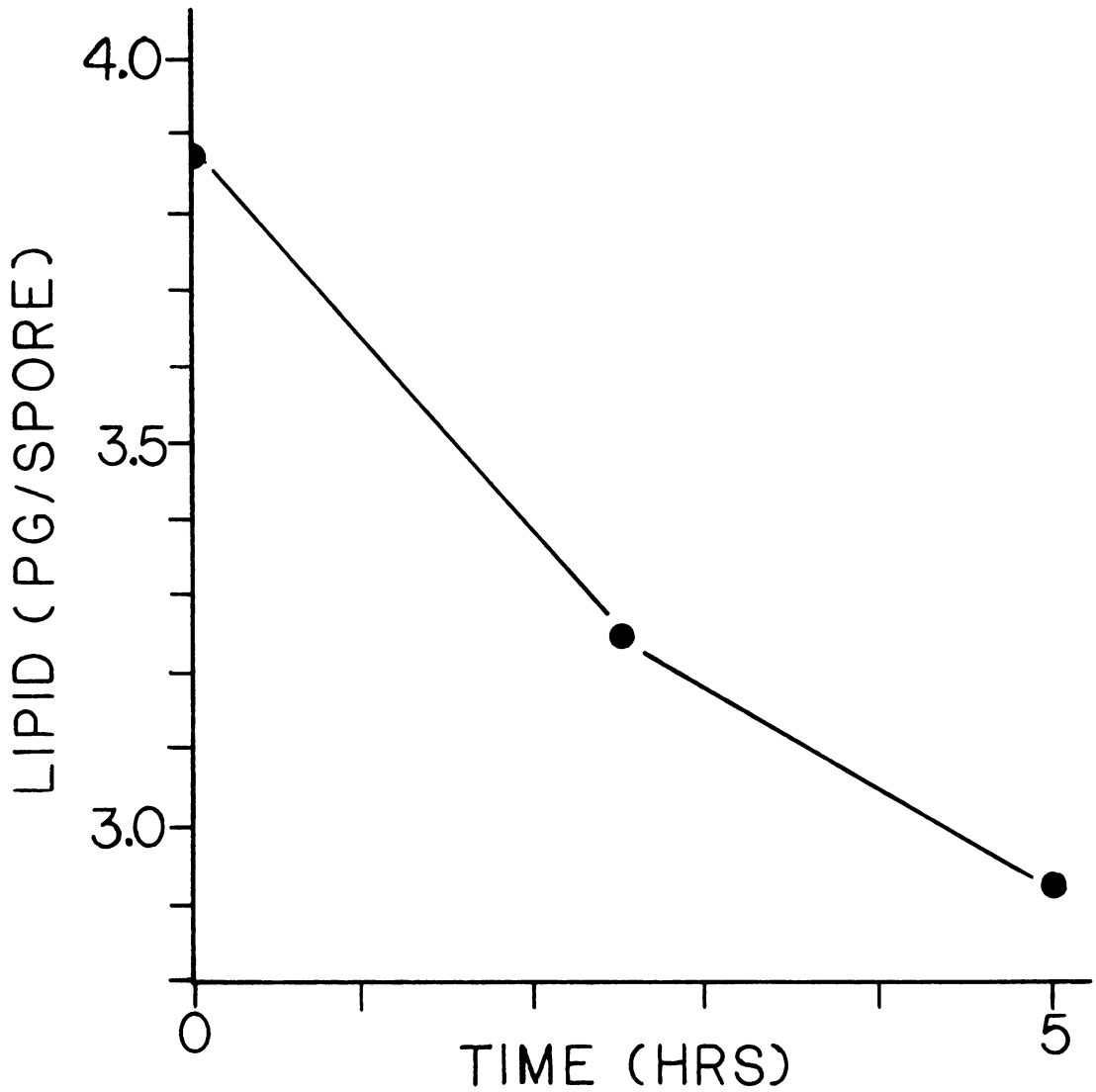

Figure 20. Total Lipid Content During Endogenous Metabolism. Points are averages of 3 experiments.

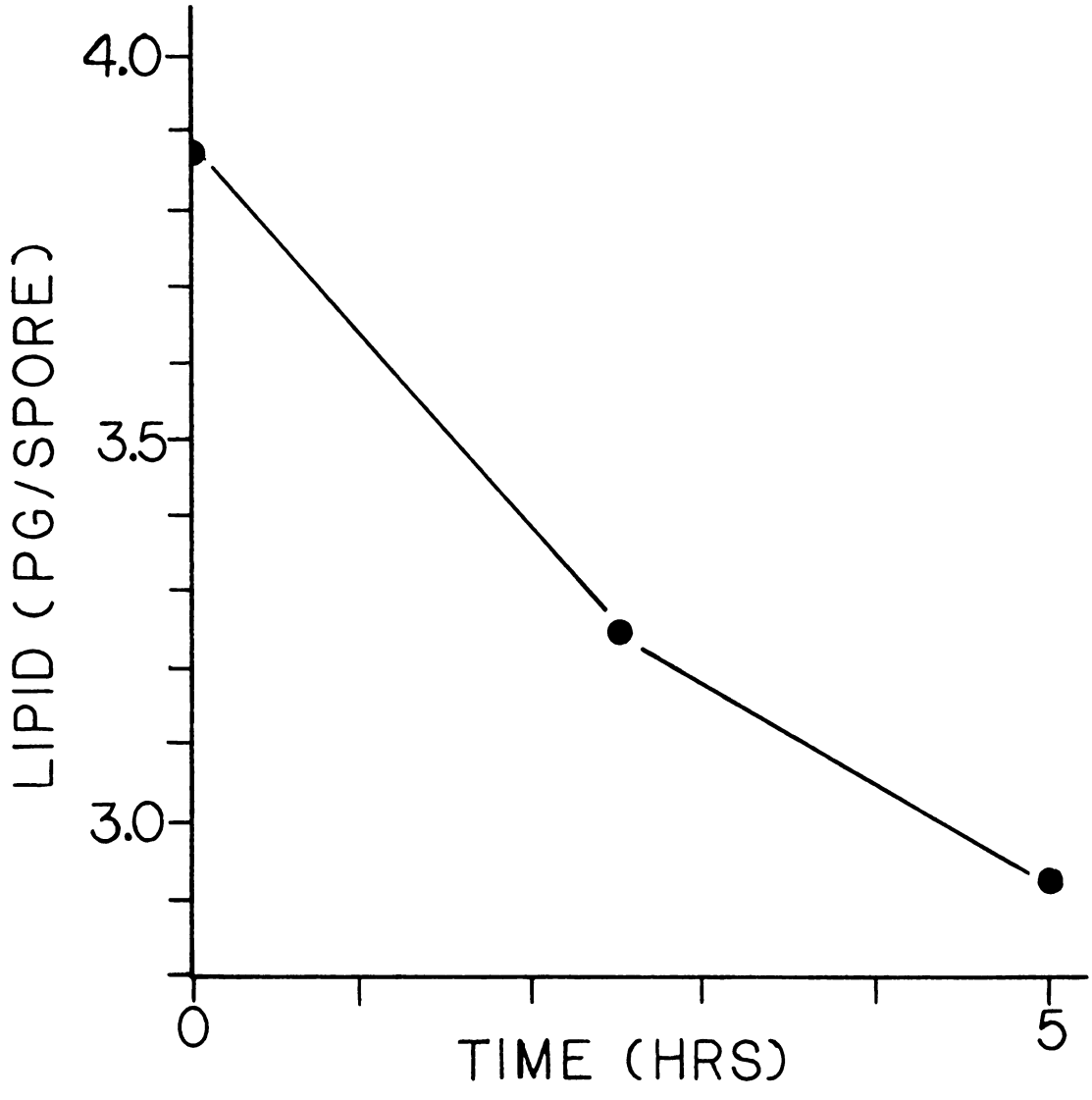

Figure 20. Total Lipid Content During Endogenous Metabolism. Points are averages of 3 experiments.

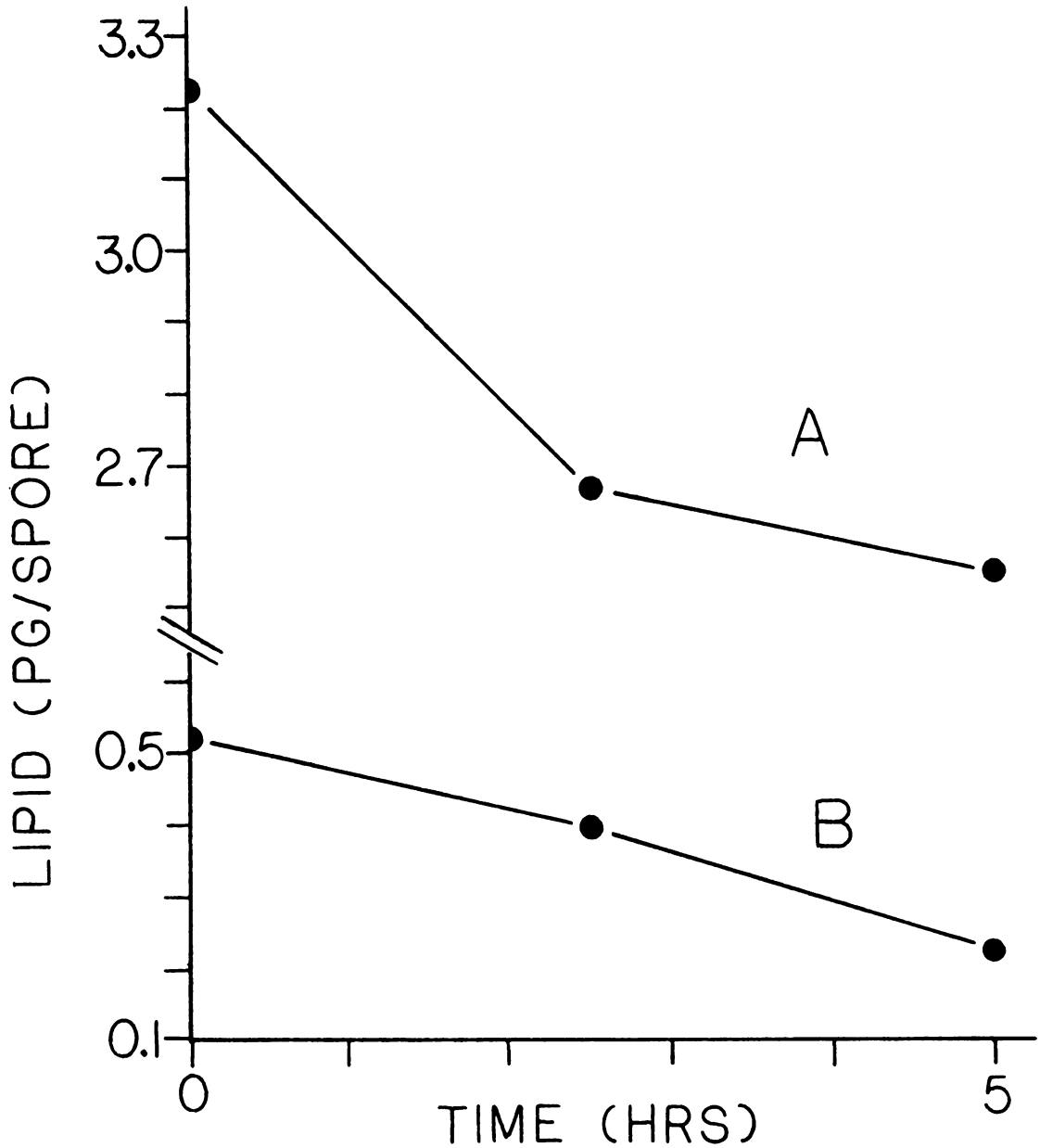

Figure 21. Neutral- and Phospho- Lipid Content During Endogenous Metabolism. Curve A: loss of phospholipid, estimated from phosphorus content. Curve B: loss of neutral lipid, estimated from acyl ester content of total- minus phospho- lipid fractions. Points are averages of 3 experiments.

The loss in phospholipid paralleled the total lipid loss in its greater initial decrease and subsequent leveling off, while the triglyceride fraction showed a more linear but smaller loss during the entire incubation. While the phospholipid was more than 6 times the triglycerides, its loss was only a little more than twice as much in the 5 hour incubation.

Although other lipid components were not measured, the possibility that they would significantly change the conclusions is not very likely. The difference between the total lipid and the sum of the phospholipid and triglyceride fractions remained fairly constant at 0.15 pg/spore and, therefore, these components could only account for ca. 4% of the total lipid.

TABLE 12. Quantities of Phosphorus and Acyl Ester in Lipid.*

Time (hrs.)	0	$2\frac{1}{2}$	5
µm P/spore (x 10^9) in total extract	4.2	3.6	3.3
µm P/spore (x 10^9) in p-lipid fraction	4.2	3.4	3.3
µeq acyl ester/spore (x 10^9) in p-lipid fraction	6.6	5.8	5.2
µeq acyl ester/um P ratio	1.58	1.65	1.57
µeq acyl ester/spore (x 10^9) in neutral fraction	1.8	1.4	0.8

* Values are averages of 3 experiments.

G.) Polysaccharide. Another endogenous reserve followed in these incubations was the glycogen-like polysaccharide (BP) first isolated from plants of B. emersonii and characterized by Cantino and Goldstein

(1961). This constituent also decreased with time (Figure 22). The averages for this particular component are somewhat misleading. The initial polysaccharide content varied considerably, depending on conditions under which plants were grown and slight variations in the amount of time from harvest to incubation. For spores grown in liquid cultures, initial values varied from 1.4 to 2.3 pg/spore; for spores from PYG plates, they were almost four times higher, 5.1 pg/spore (see next section). By plotting the data as the averages of cumulative losses from experiments which had similar starting values, the results appear as in Figure 23. The three curves here represent experiments with: (i) starting values of 1.5 pg/spore, the hourly rate of disappearance remaining constant at 0.10 pg/spore, (ii) starting values greater than 2.0 pg/spore, the hourly rate of disappearance being more than twice as much at 0.21 pg/spore, and (iii) starting value of 1.9 pg/spore, the initial hourly rate being 0.20 pg/spore, but the level of BP reaching 1.5 pg/spore at two hours and the hourly rate of disappearance changing sharply to 0.10 pg/spore. This decreased rate of disappearance when a certain threshold is reached has been repeatedly observed (see Results IV and Discussion).

H.) Physiology of Plate Produced Spores. Populations of zoospores, produced from OC plants grown on PYG agar and allowed to discharge spontaneously, i.e. without induction, were used to follow some of the endogenous physiological changes which had been monitored in zoospores from liquid cultures to determine if there were any differences in activities. The higher polysaccharide content (preceding section) was the only significant difference, and even with this high initial value its rate of disappearance was close to that in spores from liquid

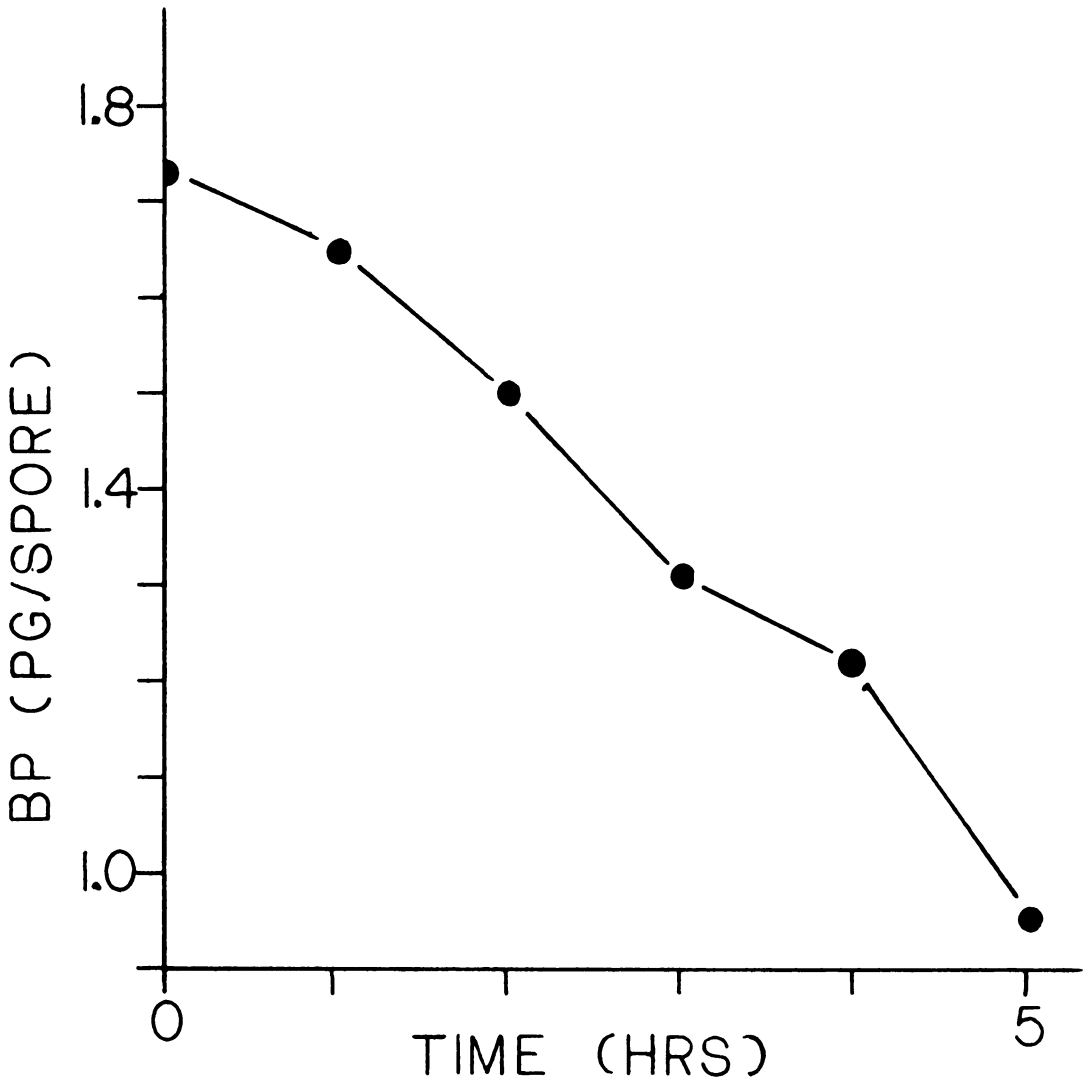

Figure 22. Polysaccharide Content During Endogenous Metabolism. Points are averages of 4 experiments.

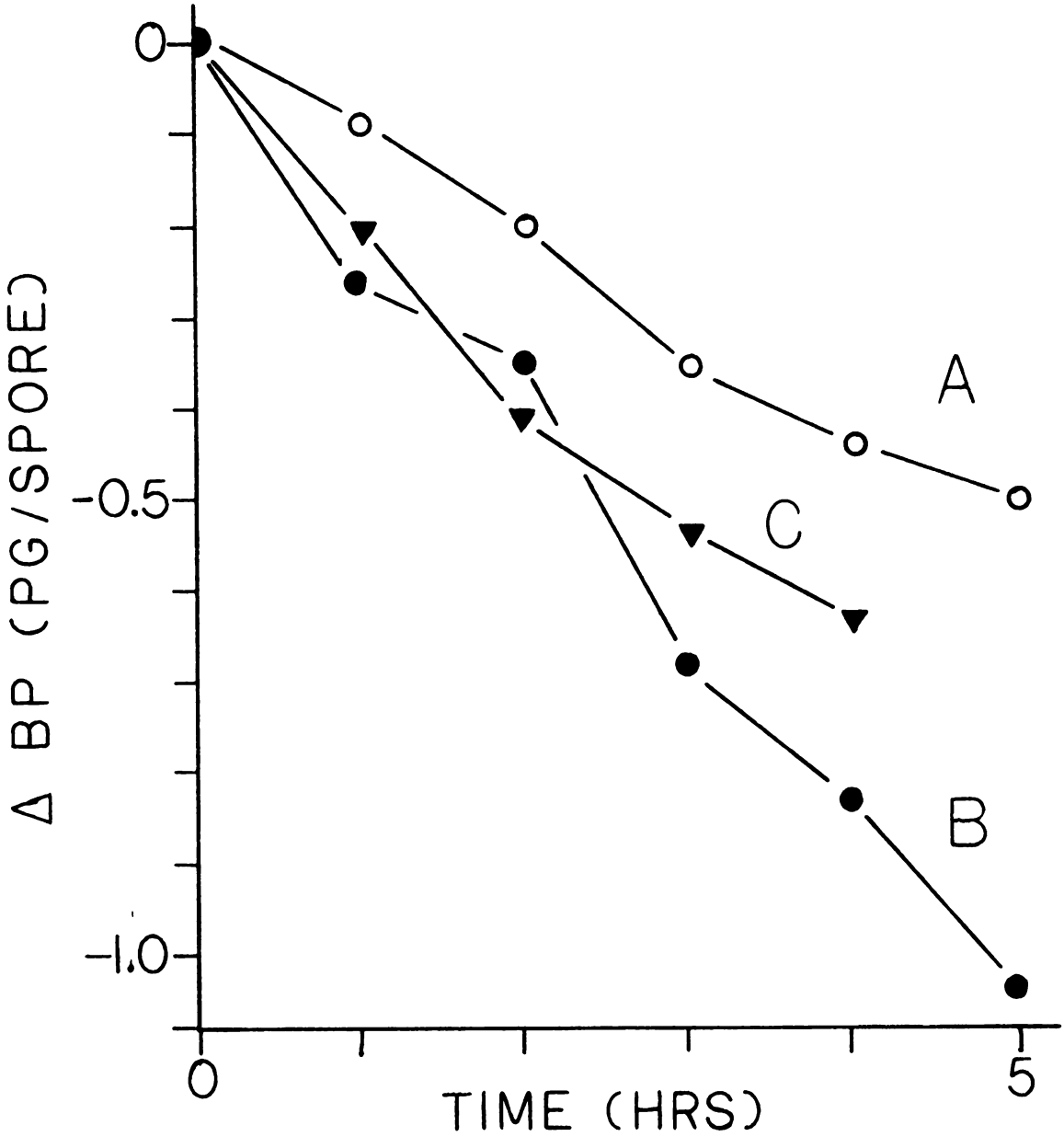

Figure 23. Effect of the Initial Amount of Polysaccharide on its Rate of Loss During Endogenous Metabolism. Curve A: initial value 1.5 pg/spore; 2 experiments. Curve B: initial value 2.0 pg/spore; 3 experiments. Curve C: initial value = 1.9 pg/spore; at 2 hours it dropped to 1.5 pg/spore; 1 experiment.

cultures which had an initial value greater than 1.5 pg/spore. The other constituents measured, protein, total N, dry weight and oxygen uptake, were not significantly different in starting values or rate of utilization from those of spores produced in liquid cultures (see Table 13).

TABLE 13. Physiological Measurements on Plate Grown Spores.

Substance measured	Initial value (pg/spore)	Hourly rate (pg/spore or Q_{O_2})
Dry weight	45.5	
Protein	24.3	0.5
Total N	4.6	0.2
Polysaccharide	5.1	0.2
Oxygen		9.4 at 0 hrs. 8.9 at $2\frac{1}{2}$ hrs.

I.) Electron Microscopy. At 0, 5 and 10 hours, spores produced from liquid cultures and incubated in the MOPS-calcium solution were sampled and examined by electron microscopy. Representative longitudinal median sections are shown in Figures 24, 25 and 26 for the three time periods. The only distinctive structural change seen as the time of incubation increased was the decrease in size and number of the lipid granules in the side body. This was strengthened by the fact that it became increasingly difficult to find lipid particles in median sections with increasing incubation time. A less definitive change was in the sb matrix, which seemed to get thinner with time. Since it is not known of what type of material the sb matrix is composed, this structural change cannot be positively correlated with any of the

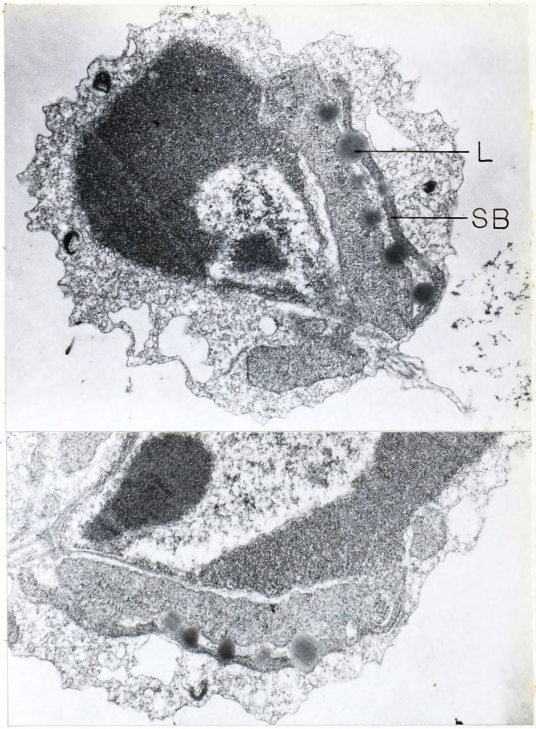

Figure 24. Sections through Zero Time Zoospores. In this and the next two figures, note especially the lipid particles (L) and the sb matrix (SB) and the changes in them. Magnification of 16, 150 x in both sections.

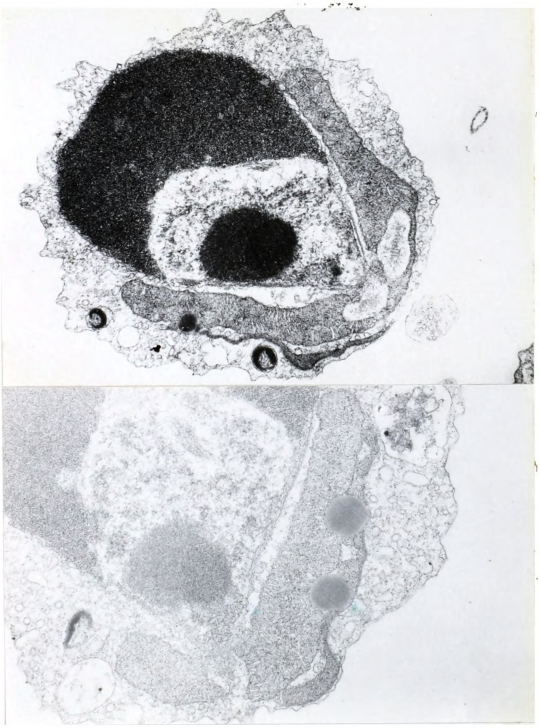

Figure 25. Sections through Zoospores After Five Hours of Endogenous Respiration. Magnification of 16, 150 x in upper; 27,500 x in lower.

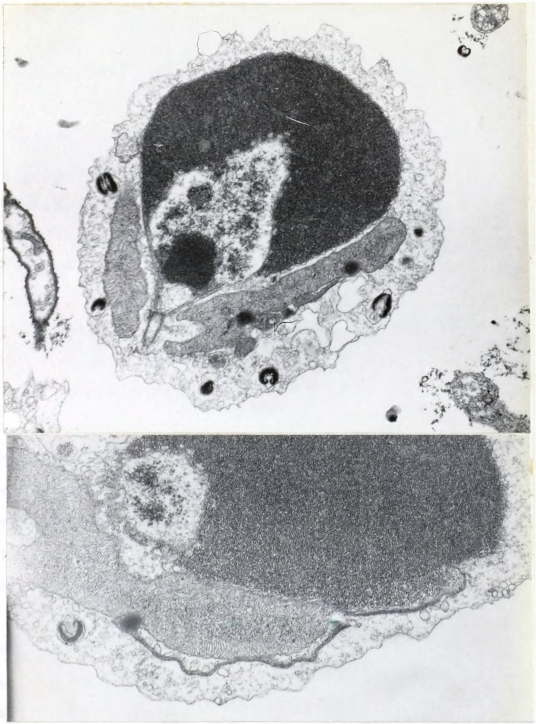

Figure 26. Sections through Zoospores after Ten Hours of Endogenous Respiration. Magnification of 16,150 x in upper; 27,500 x in lower.

chemical determinations, but it could indicate that the sb matrix is the site of the protein reserve (see Discussion).

IV. Exogenous Physiology

All previous work described in this thesis has been concerned with physiological changes in zoospores of B. emersonii which were metabolizing endogenously, i.e. no utilizable substrates were added to the media. The results of experiments in which nutrients were added will be reported here. These types of experiments are somewhat difficult to interpret for zoospores, because the addition of various chemicals can cause a proportion of the population to encyst and thus change the composition of the system. It is, therefore, difficult to determine if the effect of the added substance is on the activity measured in the nonencysted zoospore or if the substance causes the zoospore to encyst and thus change the activity measured. In most of the following experiments the %NE was 90-100, but in some cases it fell lower, especially when peptone or yeast extract was added. Nonetheless, even with these reservations in mind, the results are considered interesting and are presented here.

A.) Effect of Exogenous Substrates on Q_{O_2}. To test the effect of added nutrients on oxygen consumption, spores were produced from OC plants on PYG plates with S = 1 mM MOPS + 1 mM $CaCl_2$, pH 6.8, T = 0, and W = 1. The washed spore suspensions were placed in an oxygen electrode chamber and equilibrated until a constant rate of oxygen consumption was established. An appropriate amount of nutrient solution (generally 1% of the volume of the suspension) was added to the solution and, after the new rate of oxygen consumption was established, samples

for determination of %NE were taken. Peptone and yeast extract gave the largest increases in Q_{O_2} (cell), Casamino Acids (vitamin free) a smaller increase (Table 14). Glucose and glutamate did not increase it; in fact, glutamate inhibited it somewhat. For peptone and yeast extract, the %NE dropped to about 70, while for glucose and glutamate it remained close to 100. As mentioned previously, this drop in %NE could be the reason for the increased Q_{O_2} with the addition of these substances. Furthermore, these experiments were of very short duration (ca. 30 minutes) so that a long lag or change in metabolism might not be detected.

TABLE 14. The Effect of Added Nutrients on Oxygen Uptake.

Addition	Q_{O_2} before add.	Q_{O_2} after add.	%NE
0.1% peptone + 0.1% yeast ext.	8.3	12.6	65
0.1% peptone	8.5	9.8	72
0.01% yeast ext.	8.9	9.8	78
0.1% casamino acids	8.3	9.0	
1 mM glutamate	8.8	8.4	97
10 mM glutamate	9.5	9.1	86
1 mM glucose	8.0	8.0	95

B.) Uptake of Glucose. Spores were harvested from liquid cultures and an incubation system where S was 1 mM MOPS + 1 mM $CaCl_2$, pH 6.8, with W = 1, T = 0. Different concentrations of glucose (0.23 mM to 4 mM) were added to the spore suspensions. At time intervals, samples were removed, the spores centrifuged out of the medium, and the residual glucose

determined (Figure 27). The loss of glucose and glutamate from the medium is called uptake in this thesis, although it was not shown that these substances actually entered the cell. At concentrations above 1 mM glucose, the uptake per cell is almost three times higher (see Table 15) than at lower concentrations. The Table shows the %NE for each concentration used along with an average slope for each experiment.

In all experiments except No. 5, the %NE was close to 90. The spores in experiment 5 were given a one hour chill period to induce more encystment and thereby determine the effect of encysted spores on the uptake glucose. Since the average rate of uptake was actually lower with ca. 50% encystment, it was thought that the effect of the 10% encystment in the other experiments was insignificant and that this uptake was indeed that of the nonencysted members of the population. There always remains the possibility, however, that cold induced encysted cells behaved differently in this respect than a normal, non chill-induced population.

TABLE 15. Glucose removed from the Medium.

Experiment number	Initial conc. (mM)	Ave. hourly rate (pg/spore)	%NE
1	0.23	0.22	90
2	0.45	0.22	87
3	0.79	0.18	90
4	0.86	0.14	98
5	0.84	0.0	54
6	1.84	0.58	90
7	4.05	0.61	84

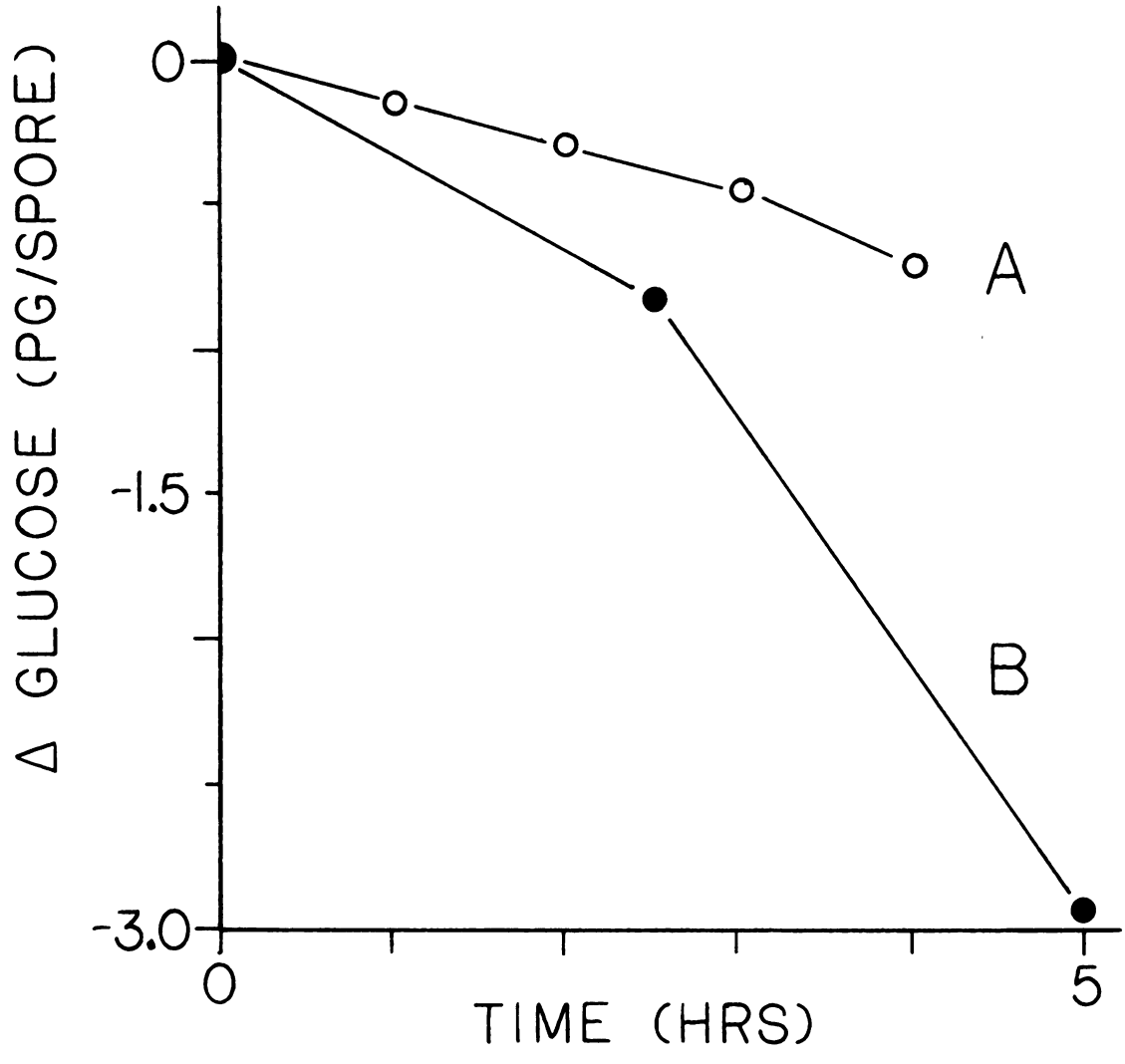

Figure 27. Effect of Glucose Concentration in the Medium on Uptake.
Curve A: 0.2-0.8 mM glucose; points are averages of 4 experiments.
Curve B: 1.4-4.1 mM glucose; points are averages of 2 experiments.

C.) Uptake of Glutamate. The disappearance of added glutamate in the incubation medium followed a pattern similar to that obtained for glucose (Figure 28). At concentrations of ca. 1 mM, glutamate disappeared 5 - 10 times faster than at concentrations below 1 mM. In fact a sharp change in uptake rate occurred between 1.0 mM and 1.1 mM glutamate. The curve for 1.21 mM in Figure 28 (Experiment 5 in Table 16) shows this quite well. It decreased at a high rate, 0.9 pg/spore for the first two hours, and then leveled off at 0.1 pg/spore when the external concentration reached 1.05 mM glutamate. Table 16 shows the %NE and average rate of uptake for each experiment. Here again, in experiment 4, spores were chilled for one hour to induce more encystment; although the %NE fell to 55, the uptake rate of glutamate also remained the same as it was with 80-90%NE.

Because protein disappeared from spores respiring endogenously, the protein in spore incubated in increasing concentrations of glutamate was followed with time. Although the results were not highly reproducible, they did indicate (Figure 29) that at concentrations below 1 mM glutamate, the protein/spore decreased in a manner similar to that of endogenously metabolizing spores. While at concentrations above 1 mM, the protein level remained fairly constant. To document all of these effects more thoroughly and to clarify their significance, however, a more intensive study is required, preferably using labelled substrates.

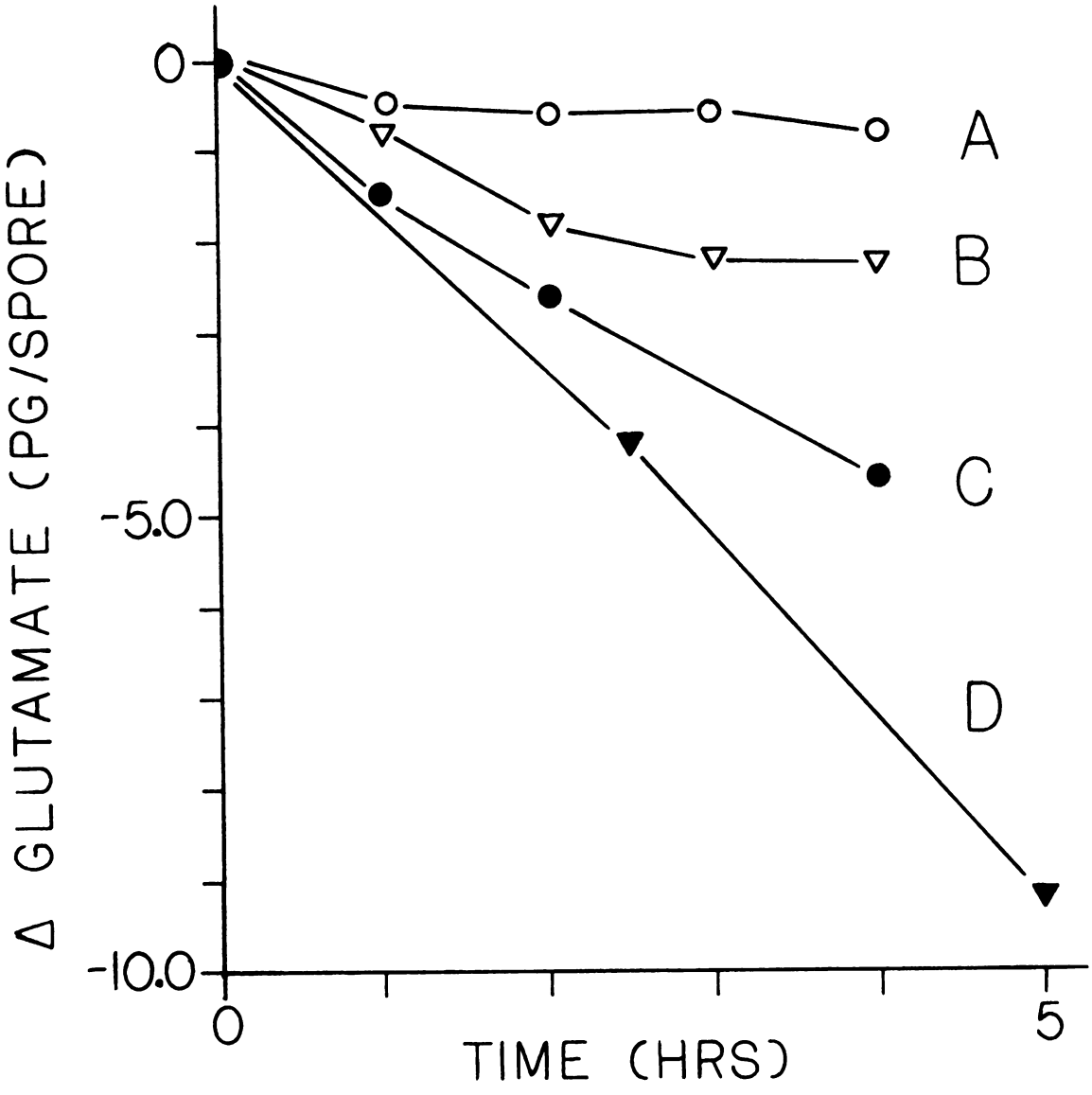

Figure 28. Effect of Glutamate Concentration in the Medium on Uptake. Curve A: 0.3-1.1 mM glutamate; points are averages of 5 experiments. Curve B: 1.21 mM glutamate; at 2 hours dropped to 1.05 mM; points are from 1 experiment. Curve C: 1.47 mM glutamate; points are from 1 experiment. Curve D: 2.08-10.7 mM glutamate; points are averages of 2 experiments.

TABLE 16. Uptake of Glutamate from the Medium.

Experiment number	Initial conc. (mM)	Ave. hourly rate (pg/spore)	%NE
1	0.32	0.20	80
2	0.56	0.20	93
3	1.04	0.20	88
4	1.01	0.20	55
5	1.21	0.90-0.10	92
6	1.47	1.10	90
7	2.10	1.60	84
8	10.7	2.00	

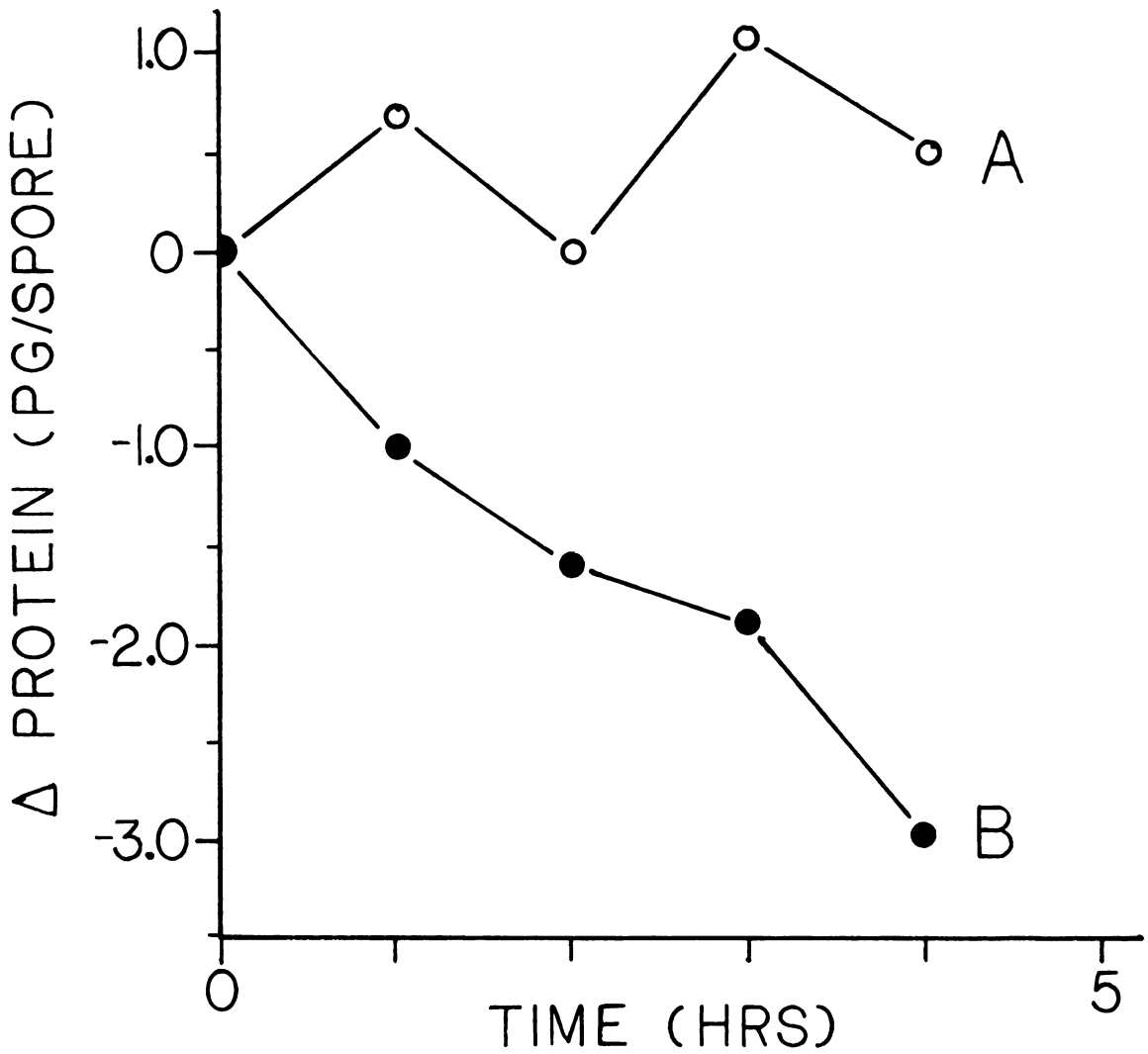

Figure 29. Effect of Glutamate Concentrations on Protein Levels. Curve A: 1.4-1.5 mM glutamate; average of 2 experiments. Curve B: 1.0-1.2 mM glutamate; average of 2 experiments.

DISCUSSION

I. Liquid Cultures.

Reasonably synchronous growth and differentiation of OC plants of B. emersonii was obtained in the liquid culture method developed here by modifying the procedure of Murphy and Lovett (1966), for the main purpose of producing zoospores. Zoospores were obtained in large enough numbers for physiological work, their average age was determined by monitoring the parent cultures. Such zoospores can be utilized to study biochemical and physiological changes. Because of the larger yields obtained, the course of events during their development can be followed at different levels of organization more easily than was previously possible.

A.) Induction and Liquid Cultures. The results obtained with liquid cultures (Results, II, A) show that, without induction, plants of B. emersonii do not differentiate into OC sporangia but seem to develop along the RS pathway. These results are not in agreement with previous work in which differentiation occurred without induction in both PYG-P (Goldstein and Cantino, 1962) and PYG-PC (Cantino and Goldstein, 1967). Although this discrepancy cannot be explained with certainty, there are several possible reasons for it.

First, in the work of Cantino and Goldstein, differentiation and discharge were not monitored long enough, since their main interest was the OC plants. In fact, generation time was defined as the time at which 5% of the plants form discharge papillae (Goldstein and Cantino,

1962) and most cultures were not followed much beyond that point (some, however, were monitored up to 50% discharge; Cantino, personal communication). Even though representative pictures (Cantino and Goldstein, 1962) show definite development into OC sporangia, the degree to which discharge occurs spontaneously in liquid medium, and its synchrony, have not been fully documented and may be quite low in comparison to induced cultures.

Second, monovalent cations (especially K^+) effect development of OC plants in both media. Using steady-state cultures in PYG, Griffin (1965) reported that 8 mM K^+ allows differentiation along the OC pathway, while at 27 mM K^+, RS plants are formed. In medium PYG-PC (Cantino and Goldstein, 1967) there is a narrow concentration range (16-22 mM) in which Na^+ is tolerated for good growth, and substituting increasing concentrations of K^+ for Na^+ inhibits plants from discharging (8mM) and eventually causes vacuolation and poor growth (16 mM). Although my cultures contained a maximum of only 7.5 mM K^+, and results were identical when Na^+ was substituted for it, these ions, possibly in combination with other factors, could have had some role in causing this lack of spontaneous differentiation.

Third, the quality of air used for aeration could have been responsible. This potential variable was not controlled since house-line air was routinely used. Toward the end of this work, however, abnormal development of plants was observed even with induction; discharge papillae were formed, but continued to elongate (frequently becoming as long as the plants) with subsequent vacuolization and death of the plants. This behavior was completely eliminated by using room air for aeration. The nature of the disrupting factor in the house-line was

not determined, and it is, therefore, possible that it was present in the aeration stream in the earlier cultures. Since the inhibition of OC sporogenesis in the noninduced cultures occurred at a different stage of development (before papilla formation) than the inhibition known to be caused by the house-line in induced cultures (after papilla formation), it does not seem probable, however, that the same agent was responsible for both effects.

Fourth, it is always possible that the organism itself has changed during continuous culture in the laboratory. Although RS stock cultures were routinely used to regenerate fresh OC lines (Materials and Methods, I, A), the possibility remains that responses to these liquid culture conditions have been altered genetically.

Although my primary goal was achieved, i.e. production of large quantities of zoospores synchronously, there remain many unanswered questions on the nature and cause of differentiation into sporangia, especially in noninduced liquid cultures. Since the medium itself, though standardized, is undefined, and there are so many other variables involved, definitive explanations for the type of behavior observed here must await more extensive experimental work and possibly the development of a defined liquid medium which can replace PYG.

B.) Light and Liquid Cultures. Light has an effect on both growth and synchrony of development of OC plants (see Cantino, 1965, and references therein). It stimulates DNA synthesis and thereby enhances synchrony of nuclear division in the early stages of growth and also at the end of the generation time (Turian and Cantino, 1959). Based on these facts, a regime of alternating light and darkness, with a final

light period coming ca. two to three hours before induction, was tested in an attempt to promote increased synchrony of nuclear division prior to induction. With the stepdown in nutrient level involved in induction, the synchrony of plants grown under this light regime is similar to that of those grown in continuous light or continuous darkness (Results, II, B). Another effect of light is to increase the generation time of the cell and thereby cause change in the amount and composition of internal constituents (Goldstein and Cantino, 1962). Since the generation times of light and dark grown cells were identical in the induced cultures, it is unlikely that any great increase in yield was obtained in light grown cultures, although this was not carefully measured.

II. Encystment.

The primary goal of my encystment studies was a practical one: to develop systems in which zoospores could either be maintained in the nonencysted state or be induced to encyst synchronously. From these studies, however, some insight might be gained as to the mechanism involved in encystment.

In order to obtain appreciable encystment, a chill period was employed. The morphological changes of zoospores during and after the chill period and the effect of the chill period on encystment in different salt solutions have been described and discussed in detail by Truesdell and Cantino (1971). In two of the salt solutions used in this thesis (sodium phosphate-calcium chloride and MOPS) and with a short chill period (T = 0.5), the amount of encystment is dependent on

population density. This relationship fits either a logarithmic or a linear plot because of the scatter, but was plotted logarithmically for a better fit at the higher population densities used (1.5×10^7 spores/ml). These results agree with those obtained with the other incubation solutions used by Truesdell and Cantino (1971) and both support and further document their proposal for an increasing self inhibition of encystment as the population density increases.

In one solution, however, (MOPS-calcium) and with a short chill period, self inhibition is not observed; in fact, it is reversed. Although the reason for this is not known, it may be due to the presence of calcium in a form which is free in solution. In the other experiments cited in this thesis and by Truesdell and Cantino (1971), whenever calcium was used, phosphate was also present. Since calcium and phosphate ions form various complexes in solution, it is possible that the effective concentration of calcium was reduced enough to prevent its effect. MOPS, which has a very low binding capacity for calcium, allows this effect to be expressed. Further studies with this kind of a system, in which the calcium concentration is lowered and substitutions made could provide very interesting insight into the mode of operation of the proposed self inhibitor, i.e. whether calcium ties up the inhibitor, prevents its formation, or competes with it for site of action.

These observations may also help explain why Soll and Sonneborn (1969) did not detect any change in encystment kinetics with a chill period. Their basic solution was much like the MOPS-calcium system, i.e. their Tris-maleate buffer does not form complexes with calcium ions, and below population densities of 6×10^6 spores/ml at which they

were working, one would not expect an effect due to the chill period (Results, I,C). This is especially significant since Truesdell and Cantino (1971) have shown that encystment induced with a chill period has a mean time of about half that induced with KCl (Soll and Sonneborn, 1969). Therefore, uncomplexed calcium ions also seem to eliminate the effect of the chill period on encystment kinetics.

It has been proposed that disruption of spore membranes may be involved in triggering encystment (Cantino et al., 1968; Soll et al., 1969). This has been supported by the changes observed during treatments with cold and sulfonic acid azo dyes (see Truesdell and Cantino, 1971, for discussion). Other observations in my work which lend support to this hypothesis concern the increasing amount of encystment obtained after a chill period when the growth temperature of the parent OC plants is increased. The growth temperature of bacterial cells affects the composition of the fatty acids in their membranes and there is a corresponding change in the permeability of these membranes during cold treatments, i.e. cells grown at lower temperatures show greater resistance to permeability changes during cold treatment than those grown at higher temperatures (Ring, 1965). This might be the case for Blastocladiella, since it is evident from Figure 10 that spores produced at 18 C do not encyst after a chill period whereas those produced at 27-29 C do so (up to 70% encystment). More work on this aspect is needed, but these results do indeed suggest that there is some change in the encystment capacity of spores formed at different temperatures. In view of the effect of varying temperatures on lipid composition in other cells (see also Sumner et al., 1969), membranes seem to be likely candidates for this effect on encystment in B. emersonii.

III. Physiology of Encysting Cells.

The physiological changes during encystment that were followed in this thesis consisted of oxygen uptake and polysaccharide disappearance in endogenously respiring cells. After spores were induced to encyst, the oxygen uptake rate increased while the polysaccharide level decreased sharply. These activities correlated closely with the encystment kinetics and it is tempting to link all three observations together. During encystment there are many rapid changes in the internal morphology of the cell (Truesdell and Cantino, 1971; Soll et al., 1969) including formation of a cell wall, dispersal of ribosomes and subsequent protein synthesis, and the change in shape of the mitochondrion. These changes probably require energy and, therefore, require that this cell carry on more respiratory activity than the zoospore. Since the rate of oxygen uptake and polysaccharide disappearance are both much greater than in nonencysted spores, it is possible that the polysaccharide is serving as the main endogenous reserve for this stage in encystment (calculations of the amount of oxygen which would be taken up by the complete oxidation of this amount of polysaccharide substantiate this). The fact that there is a slight decrease in the rate of oxygen uptake after 15-20 minutes also suggests that some endogenous nutrient has been depleted. This is, in fact, the time at which the amounts of polysaccharide has fallen to ca. 10% of its initial value and the rate of disappearance has leveled off.

It should be noted that these nutrient-poor conditions do not allow normal development beyond 30 to 40 minutes after encystment. After the cells form germ tubes, the only morphological changes detected

are formation of extensive rhizoids and eventual death of the cell (loss in refractility of the cell by phase microscopic observations comes ca. one hour after encystment). Therefore, if exogenous nutrients had been present at the time of encystment and normal development allowed, the level of polysaccharide might not have fallen to such a low level.

IV. Physiology of Nonencysted Spores.

There have been very few studies on the physiological changes which occur in zoospores of aquatic Phycomycetes. Two major reasons for this had been the difficulties in producing large quantities of zoospores synchronously, and in preventing them from encysting during experimentation. Both of these difficulties were overcome, and an examination of the nature of the changes which occur in this part of the life cycle was begun.

Zoospores of B. emersonii can swim for extended periods of time in the absence of external nutrients (Cantino and Hyatt, 1953; Soll, 1970). This indicates that endogenous reserves are important sources of energy. This was confirmed in this thesis (Results, III and IV), and it was shown that the rate of uptake of the exogenous substrates, glucose and glutamate, is very low until external concentrations exceed 1 mM. A similar situation was found for zoospores of Phytophthora drechsleri. Using concentrations of labelled sugars and amino acids below 1 mM, Barash et al. (1965) found that the motile cells released little labelled CO_2 while those which had encysted released much more. They concluded that while zoospores could utilize exogenous substrates, they probably used endogenous reserves as their main energy sources. It would appear

that in situations resembling the natural environment, i.e. when exogenous nutrient levels are low, zoospores of at least these two water molds utilize mainly endogenous storage products.

A.) Endogenous Reserves. Substances in the zoospores of B. emersonii which decrease during endogenous respiration include protein, lipid, and polysaccharide; in fact, the only major constituent analyzed which remains relatively constant is nucleic acid. Although the apparent utilization of three components seems unusual, other fungal cells have also been shown to utilize more than one endogenous substrate. These include: Neurospora tetrasperma ascospores, lipid and carbohydrate (Lingappa and Sussman, 1959); Puccinia graminis tritici uredospores, lipid and protein (Shu et al., 1954) and Verticillium albo-atrum conidia, lipid and carbohydrate (Throneberry, 1970). To my knowledge, there have been no such studies done on zoospores of other Phycomycetes. Bacteria also use a wide variety of endogenous substrates (see Dawes and Ribbons, 1964, for review).

Animal spermatozoa, which are probably more like spores of B. emersonii structurally than are spores of nonaquatic fungi, utilize mainly phospholipid as endogenous reserves (Mann, 1967), and although mammalian spermatozoa rely primarily on exogenous nutrients, those of invertebrates are thought to be chiefly dependent on their endogenous phospholipid (Higashi and Kawai, 1968; Bishop, 1962).

The lipid loss, determined analytically, in zoospores of B. emersonii was supported by electron micrographs showing a decrease in size and number of the lipid particles of the side body in spores of increasing age. Since up to 85% of the total cellular lipid is phospholipid, and

it accounts for 70% of the total decrease, the lipid particles probably contain a sizable fraction of phospholipid. The presence of acid phosphatase in the sb matrix (Cantino and Mack, 1969; L. V. Leak, personal communication) also strengthens this assumption.

Although the location of the polysaccharide utilized during these incubations was not determined, its similarity in behavior to BP on isolation suggest that it is present in the cytoplasm as the α- and β-particles observed by Lessie and Lovett (1968) in the plants and spores of B. emersonii.

Protein also found by chemical determination to decrease during endogenous respiration, was more difficult to localize. The assumption that this decrease reflected breakdown and utilization was strenghtened by the observation that NH_3-N accumulates in the medium in an amount corresponding closely to the loss in protein-N. Since protein makes up over 50% of the dry weight of this cell, it is difficult to determine whether any particular protein from any particular organelle was being used. DEAE-cellulose fractionation did not resolve this (Appendix A). However, the apparent decrease in size of the sb matrix with time, as observed in the electron micrographs, suggests that this organelle might be serving as a protein reserve, much like the lipid particles act as a lipid reserve. The seeds of some plants contain organelles which seem to serve mainly a protein (albumin) storage function to provide energy and raw materials for germination (Varner, 1966). It would not be too unreasonable to assume that the sb matrix of zoospores of B. emersonii could have an analogous function. To show this, however, further experimentation must first be carried out including the isolation and characterization of the sb matrix and protein utilized.

Finally, in support of the apparent utilization of all three endogenous reserves, the R. Q. was 0.92. Although this value could be interpreted in several ways, it does support the idea that equal quantities of lipid and polysaccharide plus ca. triple that amount of protein are being metabolized, i.e. the weighted average of the R. Q.'s for these three substrates is close to 0.9.

B.) Oxygen Uptake. The endogenous rate of oxygen uptake by nonencysted spores remains constant when measured manometrically, but is lower and decreases slightly over a five hour incubation period when determined with the oxygen electrode (Results, III, B). Since CO_2 inhibits oxygen uptake slightly (Results, I, A), it is thought that the CO_2 in the oxygen electrode vessels caused this decrease in rate. Even though the suspensions from which samples were taken for electrode determinations were aerated and stirred, certainly there was more CO_2 present than in the Warburg flasks with KOH in their center wells, it is also possible that increasing amounts of CO_2 dissolved during the incubation period, thereby also accounting for the decrease in Q_{O_2} (cell) from 9.4 to 7.8. This seems especially likely if all the derivatives of CO_2 in solution and the rise in pH of the medium are considered.

It should also be noted that the Q_{O_2} values of 8-9 reported here for zoospores are much lower than the previously reported values of ca. 100 (Cantino and Lovett, 1960; McCurdy and Cantino, 1960). There are several factors to consider in making comparisons: (i) the temperatures of incubation were different, values reported here having been generally obtained at 22 C, while Cantino and Lovett used 30 C.

However, assuming that the linear relationship between oxygen uptake and temperature (Figure 3) continues to 30 C, extrapolation would give a value for Q_{O_2} (cell) of only 25 with populations in which %NE is between 60 and 90; (ii) the method of expressing oxygen uptake rates was different, values here being reported as Q_{O_2} (cell) based on 10^7 cells instead of the dry weight used by Cantino and Lovett. However, their value for dry weight of 1.14×10^7 spores/mg makes the two ways of expressing Q_{O_2} almost identical; and (iii) the percentage of the populations which was nonencysted was probably different. The populations were not monitored for %NE by Cantino and Lovett and therefore could have contained mainly encysted cells instead of zoospores.

The third possibility is the most likely; the Q_{O_2} increases more than twofold when the cells encyst (Results, I, B). In one Warburg experiment not reported in the Results, the conditions of Cantino and Lovett were approximated, i.e. oxygen uptake was measured manometrically at 30 C in 5 mM Na phosphate, pH 7.8, and their conversion factor for mg dry weight was used in the Q_{O_2} calculations. A Q_{O_2} of 40 was obtained, but it was determined that 93% of the spores had encysted. It is felt, therefore, that earlier determinations of the rate of oxygen consumption were actually measurements of the respiratory activity of encysted cells rather than zoospores.

Another factor to be considered in resolving this question is the depletion of endogenous reserves. The time required for all of the polysaccharide of spores produced in liquid cultures to be completely converted to CO_2 and water (assuming 100% efficiency through glycolysis and the Krebs cycle) with a Q_{O_2} of 9 is ca. 2 hours. The time for the lipid to be consumed (assuming mainly stearic and palmitic acids,

efficiently oxidized; Higashi and Kawai, 1968) would be slightly more than 5 hours. Therefore, any greatly higher rate of oxygen uptake would be unreasonable for zoospores for any extended time periods in the absence of exogenous nutrients unless their mitochondria were in some way uncoupled. This would be especially true if large quantities of lactic acid were simultaneously being produced (Cantino and Lovett, 1960).

C.) Decreasing Substrate Concentration and Change in the Rate of Disappearance. The rate of disappearance (utilization) of both external substrates (glucose and glutamate), and the internal pool of BP is related to their concentration. At low concentrations the rate of utilization is low, but once a certain threshold is exceeded the rate increases: twofold for BP, threefold for glucose, and up to tenfold for glutamate. Assuming that internal polysaccharide is utilized for energy production, the parallel behavior in all three instances suggests that the external concentration does not affect the transport into the cell directly, but rather the rate of breakdown inside the cell. Until these rates are shown to be directly related to utilization and conversion to CO_2, however, this assumption remains unverified. Preliminary results reported here, and the fact (Soll, 1970) that zoospore suspensions convert labelled glutamate to labelled CO_2 certainly suggests that this is the case. Further experimentation should resolve some of these questions and lead to a better understanding of the nature of the cellular control mechanism which could be operating here.

D.) Energy of Maintenance versus Energy of Motility. The energy necessary for the survival of a cell without growth has been called the "energy of maintenance." The processes for which cells expend most of this energy generally include resynthesis, osmotic regulation and heat loss to the environment. These processes have been studied rather extensively in bacterial systems (see Dawes and Ribbons, 1964, for review). With bacterial cells it is often difficult to distinguish between the energy required for growth and that required for maintenance. The zoospore of B. emersonii offers several advantages for studying the latter: (i) it does not grow; (ii) it has a very low level of synthesis, if any (Lovett, 1968; Soll and Sonneborn, 1971); and (iii) these activities are controlled by the cell instead of being experimentally controlled e.g. starvation methods are generally used for cells which can quickly and reversibly enter the growth phase. Presumably, the main energy requirements of the zoospore are osmotic regulation between it and the environment, osmotic regulation intracellularly, flagellar and/or amoeboid movement, and heat loss. It is also possible that energy is required to maintain the highly ordered state of the organelles in the cell, i.e. to prevent encystment.

Evidence for these requirements comes from the behavior of zoospores incubated at 4 C or less. Figure 3 (Results, I, A) shows that the rate of oxygen consumption falls close to zero when spores are incubated at 4 C. Assuming that this indicates energy production by the cell, it could be concluded that at these low temperatures the cell produces very little energy, and that changes which occur in the cell indicate that processes require energy for their continuation. Examination of zoospores incubated at or below 4 C showed the following: (i) flagellar and amoeboid

activity stops; (ii) osmotic control is lost, for the cells begin to swell to two or three times their original size and eventually lyse; and (iii) the highly ordered state of the organellar arrangement is partially lost, (especially in the side body, with dispersion of lipid particles into the cytoplasm, rounding of the mitochondrion and breakage of the backing membrane). These changes have been more thoroughly documented with light and electron micrographs elsewhere (Truesdell and Cantino, 1971; Cantino et al., 1969; Shaw and Cantino, 1969).

In order to obtain information about the relative proportions of these energy requirements, oxygen uptake experiments with flagellated and deflagellated spores were performed (Results, I, C, 4). The decrease in oxygen uptake by deflagellated spores (ca. 15% of the flagellated spore uptake) indicates that a minimum of 15% of the total energy of the cell is utilized for flagellar activity. This value is considered to be a minimum because there was not 100% deflagellation, and because only the extended portion of the flagellum was removed, while the internal apparatus might still have been consuming some energy. This correlates well with Soll's (1970) observations that deflagellated spores maintain themselves for a longer time than flagellated ones under conditions of endogenous respiration.

It should then be reasonable to assume that the rest of the energy produced endogenously (something less than 85%) would be utilized for the other energy requirements of the cell. With further experimental refinements, and possibly selective inhibitors, much more information may be obtained about the relative importance and even absolute energy requirements for other fundamental processes such as osmotic regulation and amoeboid movement in the zoospore of B. emersonii.

SUMMARY

1. Methods for the synchronous production of large quantities of zoospores of B. emersonii and the control of the encystment process in them are modified and refined.

2. Populations of zoospores exhibit a self-inhibition of encystment after a chill period in two of the systems used; in another one (MOPS-calcium), however, the nature of this effect is reversed. The possible role of calcium in these systems is discussed.

3. The rate of oxygen uptake of zoospores increases more than twofold during encystment (from a Q_{O_2} (cell) of 8-9 for zoospores to 19-21 for encysted cells). At the same time there is a sharp decrease (90% in 20 minutes) in the level of the internal polysaccharide pool.

4. Polysaccharide, lipid and protein contents of nonencysted zoospores decrease during endogenous respiration while the nucleic acid content remains relatively constant. The rate of disappearance of polysaccharide and lipid are approximately equal with the rate of protein loss being ca. three times as great. Fine structure changes include a decrease in lipid particles and a thinning of the sb matrix during endogenous respiration, thus providing indirect evidence for the localization of the lipid and protein reserves in these organelles, respectively.

5. Preliminary results with exogenous glucose and glutamate and with the endogenous pool of polysaccharide, suggest that the rate of utilization of these substrates is dependent on their concentration.

6. Zoospores which are deflagellated mechanically have an oxygen uptake rate 15% lower than those which are flagellated, indicating that at least 15% of the energy of the cell is spent in flagellar activity. Other energy requiring processes of the cell are also described and discussed.

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APPENDICES

APPENDIX A

Fractionations of Zoospore Protein

Since it has been shown in this thesis that the protein/spore decreased during endogenous metabolism, an attempt was made to determine if any specific fraction of it was being utilized. Columns of DEAE cellulose were used to fractionate soluble protein samples from zoospores which were either not incubated or incubated for five hours at 22 C. The procedure was similar to the step-wise fractionation of soluble protein used for OC plants (Goldstein and Cantino, 1962).

Initial attempts to use spores produced from the same cultures did not yield satisfactory results because of the elapsed time between preparation and fractionation on the column of the two samples. The final procedure adopted made use of zoospores produced from two 4.5 liter FYG-P cultures for the two time periods and incubated in the MOPS-calcium incubation medium. The zoospores, 4.2-4.5 x 10^9 cells, were concentrated to 15 ml in 10 mM Tris-HCl, pH 7.5, homogenized with a Branson Sonicator for four minutes at power setting 4, centrifuged five minutes at 1600 x g, and the supernatant removed and centrifuged at 10,000 x g for ten minutes. Polysaccharide was removed with B-amylase (20 minutes at 24 C), followed by dialysis against 6 liters of 1 mM Tris-HCl, pH 7.5, overnight. The preparation was centrifuged again at 2800 x g for five minutes. All operations except the B-amylase treatment were done at 0-4 C. The soluble protein was then added to a 1 x 11 cm DEAE-cellulose column, previously washed with 1 M NaCl and water, and cooled to 12-13 C. The protein fractions were eluted by

stepwise addition of 50 ml each of increasing concentrations of NaCl (.005, .0055, .007, .01, .035, .05, .065, .09, .125, .175, .4, .5, .65 and 1.0 M) and collected in 5 ml samples with an automatic fraction collector. Protein and nucleic acid in the fractions were estimated from 260 and 280 mu absorption data using a Beckman DU Spectrophotometer.

The results for the protein fractionation are given in Figure 30. The patterns for zero time and five hour spores were very similar, the only major difference occurring between fractions 45-60. Here Curve 30A dropped to zero protein while Curve 30B showed a small peak. Curve 30B also had a much larger initial peak. These patterns for zoospores were similar to those obtained for OC plants (Goldstein and Cantino, 1962), except for the peaks at fractions 40 and 50 which were much smaller and nonexistant respectively, in zoospores while being major fractions in OC plants.

Figures 31A (0 hours) and 31B (5 hours) give the pattern obtained for nucleic acid. As would be expected from the apparent stability of this component, the fractionation results were almost identical.

It was concluded that the known decrease in protein from zoospores was not reflected in these results for one or more of the several reasons: (i) the method was not sensitive enough to reveal differences; (ii) no specific protein fraction was being degraded but, rather, there was an across-the-board degradation; or (iii) the protein which was being degraded was not solubilized with the procedures used, i.e. may never have been put on the column.

For a more definitive look at this problem, a combination of differential centrifugations combined with electrophoresis of solubilized protein similar to the method used for Physarum (Jockusch et al., 1970) was considered but not initiated for lack of time.

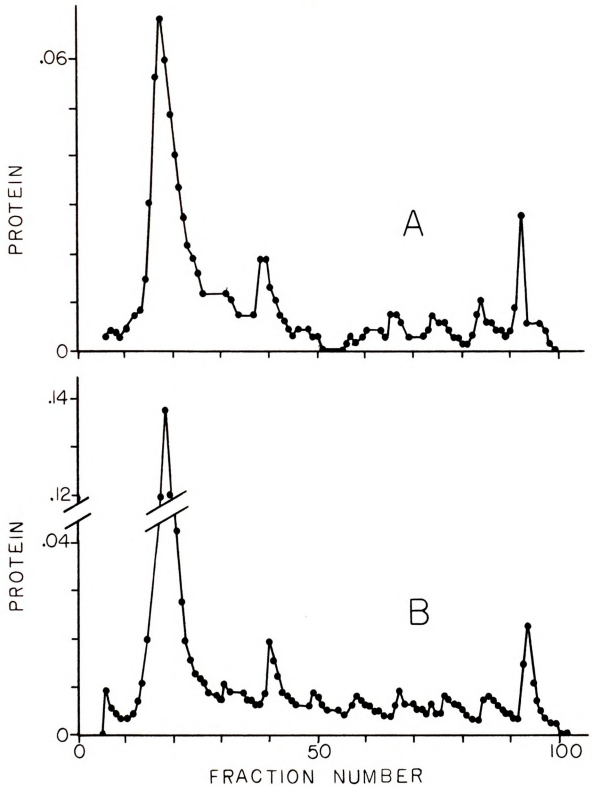

Figure 30. Protein Fractionation of Zoospores on DEAE-Cellulose. Curve A: zero time spores. Curve B: 5 hour spores. Protein is plotted as mg protein/fraction divided by total protein on the column.

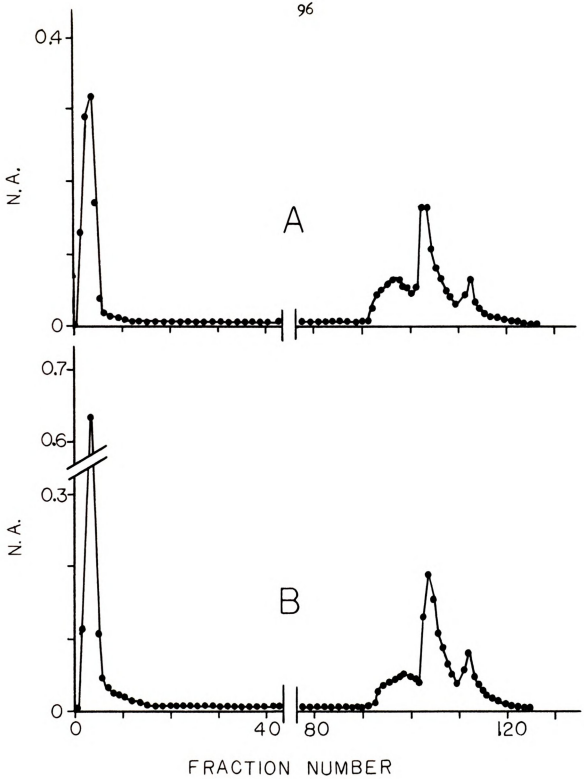

Figure 31. Nucleic Acid Fractionation of Zoospores on DEAE-Cellulose. Curve A: zero time spores. Curve B: 5 hour spores. Nucleic acid is plotted as mg nucleic acid/ml.

APPENDIX B

Attempts to Isolate Mitochondria from Zoospores

The zoospores of Blastocladiella emersonii have only one large mitochondrion which, by virtue of its position in the cell, seems to be closely linked to both the banded rootlet and flagellum, and the lipid particles and sb matrix. In order to study the energetics of zoospores more thoroughly, attempts were made to isolate and characterize these mitochondria with respiratory methods.

Several homogenization and isolation procedures were used. They were screened in preliminary fashion for their effect on mitochondria by observing isolated fractions with phase microscopy and by determining the respiratory control ratio (maximum rate of oxygen consumption with ADP additions/rate of oxygen consumption after the ADP has been used up; Chappell and Hansford, 1969). The results obtained with the substrates employed, succinate and α-ketoglutarate, indicated respiratory control with both NAD-linked and flavin-linked oxidations.

Homogenization. Breakage of spores without damaging mitochondria was and still is the first major obstacle. Since the mitochondria are so large in relation to the size of the cell, techniques which gave adequate cell lysis also damaged the mitochondria, whereas techniques which gave mitochondria that appeared intact microscopically did not rupture many cells.

Initially, various modifications of the osmotic shock technique for isolating yeast mitochondria (given in Chappell and Hansford, 1969) and gamma particles from zoospores of B. emersonii (Myers and Cantino,

1971) were used. Both sucrose and mannitol were employed as the osmotic agent, and the osmotic changes required for cell lysis were varied. In all instances where cell breakage was high enough to permit isolating mitochondria, the respiratory control ratio was close to one (i.e. no effect of added ADP) with both substrates.

The method of homogenization used by LeJohn et al. (1969), which consisted of forcing spores through a 27 gauge needle 3 times in a solution of 0.25 M Sucrose, 0.1 mM EDTA and 5 mM K phosphate, pH 7.1, also did not give higher respiratory control ratios, and in several instances did not yield good cell breakage.

A modification of the above method finally developed gave the best results. A very concentrated spore suspension (ca. 5×10^8 spores/ml) in 0.73 M mannitol, 10 mM KCl, 10 mM K phosphate, pH 6.8, 2 mM EGTA, and 1% bovine serum albumin was passed through a 27 gauge hypodermic needle twelve times to obtain good breakage of the spores. Phase microscopic observations of the mitochondria showed little damage, although they were spherical and many still had lipid particles attached.

Isolation. Previously published centrifugations for obtaining mitochondria from zoospores (LeJohn et al., 1969; Horgen and Griffin, 1969) were at higher g-values and for longer time periods than necessary to pellet intact isolated mitochondria. The final centrifugation sequence developed involved: (i) to pellet large debris, whole cells, and mitochondria; 3500 x g for four minutes; (ii) to separate whole cells and intact nuclear apparatus from mitochondria; 400 x g for eight minutes with one wash of the resuspended pellet; and (iii) to pellet mitochondria and eliminate debris; 3500 x g for four minutes. After this procedure the mitochondrial fraction was fairly clean microscopically.

It was then resuspended in the homogenization medium and used in the subsequent determinations of the respiratory control ratios. All procedures to this point were done at 0-4 C. Mitochondrial incubations were at 22 C.

Results with the Final Procedure. Representative tracings from the recorder chart are reproduced in Figure 32-34. Figures 32 and 33 show the results with addition of NAD-linked substrates, glutamate and α-ketoglutarate. With these substrates, there was a low level of respiratory control with ratios of 3.5 and 2.5 respectively. With the flavin-linked substrate, succinate (Figure 34), there was no respiratory control. This, together with the fact that the mitochondrial preparations utilized NADH (Figure 34), led to the conclusion that many mitochondria were damaged or broken (Chappell and Hansford, 1969; Lehninger, 1951).

Conclusions. From the preliminary results obtained with the final procedure, it was decided that too much time would have to be spent in improving the system for the amount of information that could be obtained. There were several possible explanations for the lack of respiratory control with succinate (see Chappell and Hansford, 1969), but the most likely seemed to be that due to the large size of these organelles, they were damaged in the initial homogenization procedure. Until a gentler method of cell disruption can be devised, this problem will probably continue to reoccur.

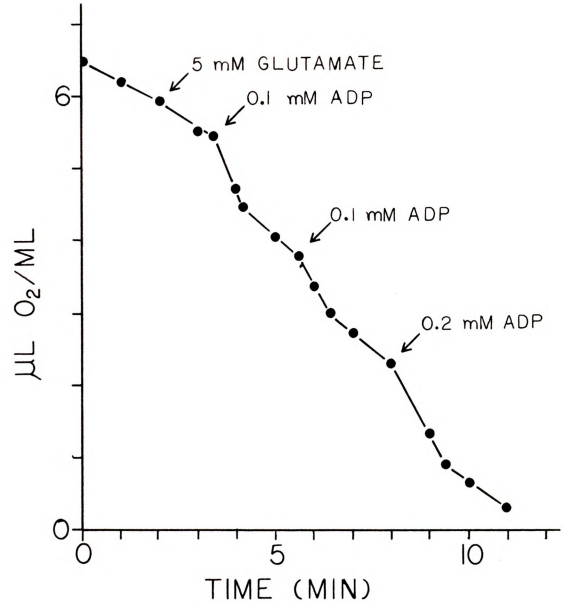

Figure 32. Oxygen Uptake by Mitochondrial Fraction in Glutamate. Mitochondrial fraction equilibrated to constant endogenous rate, and zero time taken at this point. Additions are shown on the figure.

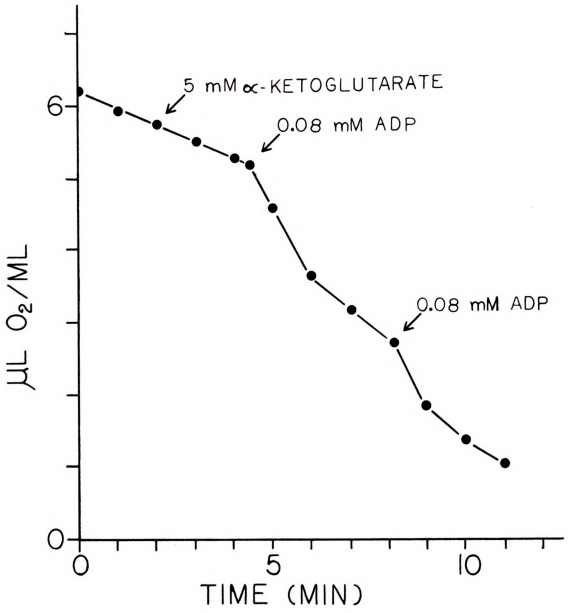

Figure 33. Oxygen Uptake by Mitochondrial Fractions in ∝-Ketoglutarate. Mitochondrial fraction equilibrated to constant endogenous rate, and zero time taken at this point. Additions are shown on the figure.

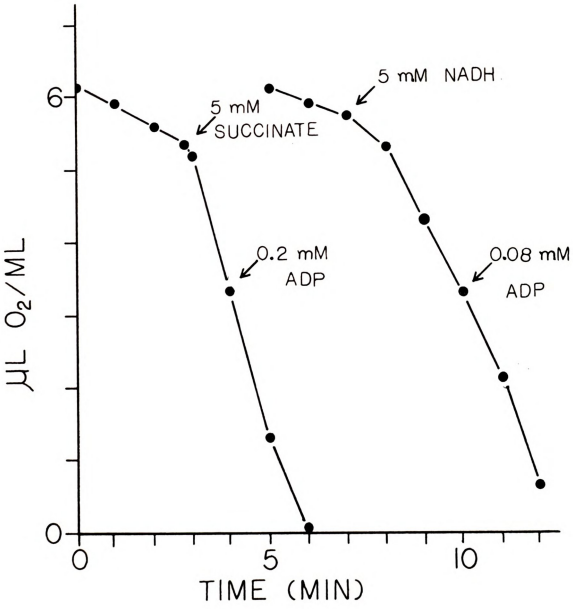

Figure 34. Oxygen Uptake by Mitochondrial Fractions in Succinate and NADH. Mitochondrial fractions equilibrated to constant endogenous rate, and zero time (5 minutes for NADH) taken at this point. Additions are shown on the figure.