THE NATURE AND FUNCTION OF THE MITOCHONDRION-LIPID-SYMPHYOMICROBODY COMPLEX IN THE ZOOSPORE OF BLASTOCLADIELLA EMERSONII

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ABSTRACT

THE NATURE AND FUNCTION OF THE MITOCHONDRION-LIPID-SYMPHYOMICROBODY COMPLEX IN THE ZOOSPORE OF BLASTOCLADIELLA EMERSONII

By

Gary Lynn Mills

The zoospores of Blastocladiella emersonii possess an elaborate, tightly organized, membrane bound assemblage of organelles. One especially prominent component is the side body complex. This complex consists of a single mitochondrion, a number of discrete lipid globules and a single-membrane-bound structure, once named the SB matrix. A study was made of the lipid composition of the zoospores, the lipid changes during development and the chemistry or enzymology of the individual components of the side body complex. The specific purpose was to try to obtain a better understanding of the functional nature of this group of organelles.

The zoospores of B. emersonii, when derived from cultures grown on solid media, contained about 11% total lipid. This lipid was separated chromatographically on silicic acid into neutral lipid (46.6%), glycolipid (15.8%), and phospholipid (37.6%). Each class was fractionated further on columns of silicic acid, Florisil, or diethylaminoethyl-cellulose, and monitored by thin-layer chromatography. Triglycerides were the major neutral lipids, mono- and diglycosyldiglycerides the major glycolipids, and phosphatidylcholine and phosphatidylethanolamine the major phospholipids. Other neutral lipids and phospholipids detected

were: hydrocarbons, free fatty acids, free sterols, sterol esters, diglycerides, monoglycerides, lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidic acid, phosphatidylserine and phosphatidylinositol. Palmitic, palmitoleic, stearic, oleic, γ-linolenic and arachidonic acids were the most frequently occurring fatty acids. When B. emersonii was grown in [1,2-^{14}C]-acetate-labeled liquid media, lipid again accounted for 11% of both the mature plants and the zoospores released from them. The composition of the lipid extracted from such plants and spores was also the same. However, it differed markedly from that of the lipid in spores harvested from solid media, consisting of 28.3% neutral lipid, 12.0% glycolipid, and 59.0% phospholipid. The major lipids were the same as those derived from plate grown cultures.

The lipid composition of swimming spores, cysts and five hour germlings was also established. Spores utilize triglycerides first, then phospholipids. Upon encystment all glycolipids decreased, while in germlings the phospholipids, monoglycerides and sterol esters exhibited a marked increase.

Lipid globules were isolated and characterized both chemically and morphologically. They were composed mainly of triglycerides and free sterols, the combination accounting for over 90.0% of the total weight of the globules. Smaller amounts of diglycerides, carotenoids, fatty free acids, phospholipids and protein were found. No sterol esters or monoglycerides were detected. Morphologically, the isolated lipid globules resembled the lipid globules in situ. They were spherical, 0.4 μm to 1.5 μm in diameter and lacked a trilaminar membrane.

Since the SB matrix lies in close proximity to the lipid globules which are primarily triglycerides, and since the triglycerides decrease as the spores swim, the SB matrix might be functioning as a microbody. Microbodies can be identified ultracytochemically with the diaminobenzidine (DAB) catalase test. When zoospores and sporangia of B. emersonii were incubated in the DAB reaction mixture both the SB matrix in the zoospores and the sb granules in the sporangia exhibited a catalase positive reaction. The two types of organelles are ontogenetically interrelated; the sb granules are microbodies, and these give rise to the symphyomicrobody (formerly the SB matrix) by symphyogenesis.

Microbodies were isolated from sporangia of B. emersonii. They had a mean buoyant density of 1.222 g/cm^3 after centrifugation through a linear sucrose gradient, and contained catalase, isocitrate lyase and malate synthase activities. Symphyomicrobodies were also isolated from zoospores. They had a mean buoyant density of 1.292 g/cm^3, hence an increase in density accompanied the formation of symphyomicrobodies by symphyogenesis. The spores single mitochondria had a buoyant density of 1.219 g/cm^3. Statistical data are provided for starting levels and purification of symphyomicrobody and mitochondrial enzyme markers.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF NON-STANDARD ABBREVIATIONS

Phospholipids

LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine

Neutral Lipids

DG	Diglyceride
FFA	Free fatty acid
FS	Free sterol
HC	Hydrocarbon
MG	Monoglyceride
SE	Sterol ester
TG	Triglyceride

Glycolipids

DGDG	Diglycosyldiglyceride
MGDG	Monoglycosyldiglyceride
PGDG	Polyglycosyldiglyceride

INTRODUCTION

In 1949, a new species of the Chytridiomycete, Blastocladiella, was discovered in a fresh water pond on the campus of the University of Pennsylvania and subsequently described (Cantino, 1951). Two years later it was designated a new species, Blastocladiella emersonii (Cantino and Hyatt, 1953), and since that time this fungus has become an important experimental organism for studying morphological, physiological and developmental interrelationships (see reviews: Cantino and Lovett, 1964; Cantino, 1966; Cantino et al., 1968; Truesdell and Cantino, 1971; Lovett, 1975; Cantino and Mills, 1976).

The life cycle of B. emersonii is rather simple although variations do occur (Hennessy and Cantino, 1972; Cantino and Myers, 1974). The fungus produces posteriorly uniflagellated zoospores. During their existence, these zoospores undergo mainly catabolic activities, very few synthetic processes having been detected (Soll and Sonneborn, 1971; Suberkropp and Cantino, 1973; Lovett, 1975). The zoospores round up, retract their flagella and encyst, a process involving dedifferentiation accompanied by striking ultrastructural changes (Soll et al., 1969; Truesdell and Cantino, 1971) and the production of a cell wall (Myers and Cantino, 1974). After a short time the cysts produce rhizoids and become germlings. The germlings undergo nuclear division and exponential growth and generate plants bearing multinucleate sporangia. Exponential growth culminates at the time of papillae formation, after which the sporangia undergo cytodifferentiation with formation of zoospores. The zoospores are released via the exit papillae and the cycle is repeated. No sexual stage is known.

The organelle arrangement in the unflagellated zoospore is highly ordered (Cantino and Mills, 1976). Each cell contains: a nuclear apparatus consisting of the nucleus, nucleolus and the nuclear cap, which houses most of the spore's ribosomes; a kinetosome-rootlet-centriole complex situated at the posterior end of the nucleus, and continuous with the flagellar axoneme; cytoplasmic gamma particles which are involved in cell wall synthesis; and the side body complex which is made up of several organelles. It is this latter group of organelles to which this thesis is directed -- the aim being to try to understand the function of this side body complex.

The history of the side body complex is lengthy and there has been much confusion about the terminology. Stüben (1939) was the first to observe, by means of light microscopy, what he called the "seitenkörper", i.e. the side body, in zoospores of other species of the Blastocladiaceae. Couch and Wiffen (1942) identified one of the components of the side body as a group of fat bodies. It was not, however, until 1963 that the true nature of the side body was determined. Cantino et al. (1963) and Lovett (1963) showed via electron microscopy that the side body complex consisted of the mitochondrion, lipid granules and particles of unknown material. These unidentified particles were later labeled "sb granules" (Lessie and Lovett, 1968). The sb granules were at first treated as separate entities; however, Cantino and Truesdell (1970) using serial sections showed that the sb granules were segments of a larger continuous structure; the latter was labeled the "SB matrix".

The side body complex is located in the posterior portion of the zoospore. The lipid granules are dispersed along the outer surface of

the long "arm" of the mitochondrion. Molded against them is the SB matrix, an organelle bound by a unit membrane that has a granular to amorphous texture of moderate electron density. A sheet of double membrane, the backing membrane, covers the whole complex of organelles and is continuous with the outer unit membrane of the nuclear apparatus.

Within the past few years, structural aggregates resembling either the whole side body complex or just the SB matrix-lipid grouping have been seen in zoospores of Blastocladiella brittanica (Cantino and Truesdell, 1971) and other Chytridiomycetes. Especially noteworthy is the side body complex described by Martin (1971) for the zoospores of Coelomomyces punctatus, wherein lipid globules are positioned between the mitochondrion on one side and a unit membrane bound SB matrix on the other. A somewhat comparable arrangement occurs in the zoospores of Coelomomyces psorophorae (Whisler et al., 1972) and Coelomomyces indicus (Madelin and Beckett, 1972).

Similarly, something resembling a side body complex was observed in the zoospores of Allomyces (Fuller and Olson; 1971), where they called the membrane bound body a "Stüben body", a term which was thought to be more general. Olson (1973) concluded later, using serial sections, that the side body complex in the meiospores of Allomyces had a three dimensional organization resembling the one in B. emersonii. Stüben bodies were also reported to occur in Phlyctochytrium arcticum (Chong and Barr, 1973), and zoospores of Harpochytrium hedinii (see Travland and Whisler, 1971) possess a tight association of lipid-like globules, a single "rumposome", and profiles of electron dense granular material resembling the SB matrix in B. emersonii.

The important question is: what is the function of these various bodies which are enclosed by a single membrane, composed of a moderately

electron opaque substance, and closely associated with lipid bodies and mitochondria? Recently a number of people studying Chytridiomycetes have concluded, based strictly on morphological criteria, that these organelles are microbodies. McNitt (1974) described a rumposome-lipid-microbody complex in Phlyctochytrium irregulare. Chong and Barr (1974) observed microbodies in Entophlyctis conferrae-glomeratae, microbody-lipid complexes in Rhizophydium patellarium and microbody-lipid-mitochondrial complexes in Catenaria anguillulae. Held (1975) observed a tight association of lipid globules and microbodies which were surrounded by a backing membrane in Rozella allomycis. In each of the above cases, however, identification of these unit membrane-bound organelles as microbodies was entirely based on morphology. Because these organelles were not studied chemically or enzymologically, their functional nature remained unknown.

Over the past few years, a large literature about microbodies has accumulated. These organelles are bound by a unit membrane and have been found in animals, plants, protozoa, algae and fungi (DeDuve, 1969; Hruban and Rechcigl, 1969; Tolbert, 1971; Vigil, 1973; Richardson, 1974; Frederick et al., 1975). Microbodies are 0.3 μm to 1.5 μm in diameter, generally are associated with lipid bodies and/or endoplasmic reticulum and have a fine granular matrix of varying electron density. Physiologically, microbodies have been classified as peroxisomes or glyoxysomes (DeDuve, 1969). Peroxisomes play a role in glycolate metabolism and photorespiration (DeDuve, 1969; Tolbert, 1971). Glyoxysomes function in the conversion of lipids to carbohydrates (Beevers, 1969; Richardson, 1974).

Morphologically, the SB matrix in the B. emersonii zoospore resembles a microbody, and it is always found in close association with lipid

bodies---a common characteristic of glyoxysomes. However, the SB matrix is some 3-4 times larger than most microbodies, and the zoospores do not possess an endoplasmic reticulum (Cantino et al., 1963), although an association between sb granules and endoplasmic reticulum may occur during zoosporogenesis (Lessie and Lovett, 1968).

If the SB matrix is a microbody, it might be functioning as a glyoxysome. Glyoxysomes play a central role in gluconeogenesis by virtue of their capacity to utilize lipids *via* β-oxidation, and to mediate the formation of succinate *via* isocitrate lyase and malate synthase by way of the glyoxylate cycle. Succinate then finds its way to mitochondria where it is further metabolized. The net synthesis of carbohydrate from lipid is therefore dependent on the metabolic interplay between glyoxysomes and mitochondria. The association of components in the side body complex, i.e. the lipid SB matrix and the single mitochondrion, seems to be an ideal arrangement for the metabolism of lipid by a glyoxysomal-type particle.

It has been known for some time that the zoospores of *B. emersonii* contain at least some of the glyoxylate cycle enzymes. Isocitrate lyase was detected in zoospore homogenates and purified some 52 fold from zoosporangial preparations (McCurdy and Cantino, 1960). Suberkropp and Cantino (1973) also found that the total lipid decreased as the zoospores swam. These data, along with the morphological characteristics, suggest that the SB matrix may be a glyoxysome.

If the SB matrix is a glyoxysome, what is its function and what is its relationship to the other organelles in the side body complex? In this study, I have attempted to elucidate some of the answers to these questions in two ways. First, the lipid composition of the zoospores was determined,

and changes in these lipids were followed during different developmental stages. Second, the individual components of the side body complex were isolated and partially characterized.

I

Lipid Composition of the Zoospores of *Blastocladiella emersonii*

Except for a reference to Katsura (1970), cited by Gay et al. (1971), which we have not seen, apparently the only available data on the lipid content of fungal zoospores, as estimated by direct chemical analysis, are to be found in two recent reports on the water mold *Blastocladiella emersonii* (Cantino and Hyatt, 1953). The first of these (Suberkropp and Cantino, 1973) established changes in the quantity of lipid/cell during endogenous metabolism of swimming zoospores; the second (Smith and Silverman, 1973) provided a preliminary description of changes in lipid composition after zoospore germination. We have been investigating the lipids in certain organelles in these zoospores (for a review of their structure, see Truesdell and Cantino, 1971), for which the composition of total cell lipid constitutes an essential reference point. In this communication, we characterize the whole spore lipid of *B. emersonii*, and provide some comparative information about the lipid content of the plants from which such spores are derived.

MATERIALS AND METHODS

Production of zoospores on a solid medium. The original strain of *B. emersonii* (Cantino and Hyatt, 1953) was grown on peptone-yeast extract-glucose (Difco; PYG) agar by inoculating with 4×10^5

spores per standard Petri plate and culturing at 22°C in the dark. Zoospores were obtained about 24 h later from first generation plants by flooding each plate with 5 ml of water and filtering 15 min later. After population densities were established with a model B Coulter counter, the zoospores were sedimented at 1,000 x g for 5 min. Under these conditions, the yield was approximately 4 x 10^9 zoospores per 100 plates.

Production of plants and zoospores in a liquid medium. PYG broth cultures were prepared, inoculated, and induced to release zoospores at 22°C, by the method of Myers and Cantino (1971). [1,2-^{14}C] sodium acetate (New England Nuclear Corp.: 50 μCi) was added 9 h after inoculation, the final concentration being 5 x 10^{-4}M. For studies of zoosporangial lipid, thalli were harvested either just before zoospore cleavage or after zoospores had been cleaved but not released (ca. 23 h after inoculation); for studies of zoospore lipid, spores were collected about 1 h later.

Extraction of lipid. Spore pellets were washed with water, sedimented, sonically treated (30 s, 80W), and extracted at room temperature with chloroform-methanol (2:1, v/v) overnight. Additional extractions did not increase yields. The spore homogenate was filtered through a coarse, fritted-glass Büchner funnel, and the filtrate was evaporated to dryness under N_2.

Nonlipid contaminants were removed with Sephadex G-25 by the method of Rouser and Fleischer (1965).

Column chromatography. Lipids were fractionated into neutral, glyco-, and phospholipids on activated silicic acid (100 mesh, Mallinckrodt Chemical Co., St. Louis, Mo.). Neutral lipids were separated further on silicic acid after removal of fatty acids (Dittmer and Wells, 1969), or on 7% hydrated Florisil (Carroll and Serdarevich, 1967). Glycolipids were separated into individual components with Florisil (Radin, 1969), and phospholipids were fractionated on diethylaminoethyl (DEAE)-cellulose (Sigma Chemical Co., St. Louis, Mo.) by the method of Rouser et al. (1969).

Thin-layer and paper chromatography. Thin-layer chromatography (TLC) was used to check the purity of column fractions. Neutral lipids were separated with petroleum ether-diethyl ether-acetic acid (80:20:1, by volume) by using ITLC-SG chromatography media (Gelman Instrument Co., Ann Arbor, Mich.). Phospholipids were chromatographed on the same media with isopropanol-ammonium hydroxide (100:7, v/v); glycolipids were separated with the same solvent on ITLC-SA media. Phospholipids were also chromatographed two-dimensionally on Redi-Coats (Supelco Inc., Bellefonte, Pa.) by using chloroform-methanol-ammonium hydroxide (60:25:5, v/v) in the first direction and chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5, v/v) in the second direction.

Lipid components were visualized with ultraviolet light, I_2 vapor, or a saturated solution of K_2CrO_4 in 70% (v/v) H_2SO_4 (Skipski and Barclay, 1969). Specific sprays included $SbCl_3$ for sterols and sterol esters (Skipski and Barclay, 1969), 0.2% ninhydrin in butanol for free amino groups. Dragendorff reagent for the detection of choline-containing compounds (Skipski and Barclay, 1969), and the reagent of Dittmer and Lester (1964) for P. Glycolipids were detected with orcinol (Skipski and Barclay, 1969), phenol-H_2SO_4 (Gray, 1965), or diphenylamine (Skipski and Barclay, 1969).

Descending chromatography of water-soluble hydrolysis products was carried out on Whatman no. 1 paper. Glycerol phosphate esters were resolved with phenol-water (100:38, v/v) (White and Frerman, 1967), and the phosphate groups were detected by the salicylsulfonic acid-$FeCl_3$ procedure (Yorbeck and Marinetti, 1965) or with acid molybdate (Hanes and Isherwood, 1949). Glycolipid hydrolysis products were chromatograhed with N-propanol-ammonium hydroxide-water (6:3:1, v/v) or ethyl acetate-pyridine-water (12:5:4, by volume; Isherwood and Jermyn, 1951). Ammoniacal $AgNO_3$ was used to detect carbohydrates. The hydrolysis products were also studied by TLC. Phosphate-impregnated Chromagram sheets (Eastman; 6061 silica gel) were prepared, spotted, and, after multiple development, sugars were located thereon, all by the method of Welch and Martin (1972).

Hydrolysis procedures. Glycerol phosphate esters were prepared by deacylating the phospholipids in 0.2 N methanolic NaOH for 15 min at room temperature (Kates, 1972). The solution was partitioned against

$CHCl_3$ and the aqueous portion was neutralized with Dowex-50 (H+). The water-soluble hydrolysis products were concentrated almost to dryness under a stream of N_2 at 40°C, and used for chromatography. Phospholipids were also deacylated by mild alkaline hydrolysis at 0°C (White and Frerman, 1967). Glycolipids were hydrolyzed in 2 N HCl for 2 h at 100°C. The hydrolysate was extracted three times with petroleum ether; the HCl was removed under a stream of N_2 or by drying the samples over KOH pellets.

Analytical procedures. Lipid-P was determined after digestion of samples with 10 N H_2SO_4 by a modification of Bartlett's method (Bartlett, 1959). Total N was assayed with the microprocedure of Sloane-Stanley (1967) or by direct nesslerization with commercial (Harleco dry pack) Folin-Wu (Folin and Wu, 1919) reagent. Acyl esters were estimated by the ferric hydroxamate method (Rapport and Alonzo, 1955) with tripalmitin as a standard. Glycerol analyses (Hanahan and Olley, 1958) were based on the determination of formaldehyde produced by oxidation of glycerol with periodate by using α-glycerol phosphate as a standard. Total hexoses were determined with anthrone (Wells and Dittmer, 1963) or the phenol-sulfuric acid method (Dubois et al., 1956). Glucose was also analyzed enzymatically (Glucostat, Worthington Biochemical Corp., Freehold, N.J.), and total hexosamines were estimated by a modification (Dittmer and Wells, 1969) of the Elson-Morgan reaction.

Gas chromatography. Fatty acid methyl esters of the total lipid and the neutral, glyco-, and phospholipid fractions were prepared by saponification and extraction of fatty acids (Dittmer and Wells, 1969); the latter were methylated with BF_3 (Metcalfe and Schmitz, 1961). Methyl esters were examined with a Packard gas chromatograph model 7300 equipped with a flame ionization detector. The methyl esters were separated on a column (0.32 by 200 cm) packed with 15% Lac-2R-446 on Chromosorb W (80 to 100 mesh) operated at 187°C. The carrier gas was He; the injector port temperature was 193°C; and the detector temperature was 225°C. The esters were identified by their retention times relative to methyl ester standards.

Materials. Phospholipid standards were prepared from egg yolks. The phospholipids were extracted with chloroform-methanol (1:1, v/v), separated from neutral lipids by silicic acid chromatography, and then fractionated on DEAE-cellulose. The phospholipids were purified further by TLC and compared with published data on egg yolk phospholipids (Rhodes and Lea, 1957). Some organic reagents and most solvents were redistilled before use. Methyl esters were obtained from Supelco Inc., Bellefonte, Pa.

RESULTS

Characterization of total lipid in spores produced on solid media. Lipid extracts were fractionated on silic acid columns (Table 1). Neutral, glyco-, and phospholipid accounted for 46.6, 15.8, and 37.6%,

TABLE 1. Lipid composition of zoospores produced on solid media[a]

Fraction[b]	Percentage[c]
Neutral lipid	46.6 ± 2.2
Glycolipid	15.8 ± 2.4
Phospholipid	37.6 ± 2.5

[a]Percentages were established gravimetrically after the lipid had been fractionated on silicic acid columns (2 by 8 cm).

[b]Fractions were eluted successively with 150 ml of chloroform, 100 ml of acetone, and 150 ml of methanol.

[c]Means and standard deviations for four experiments.

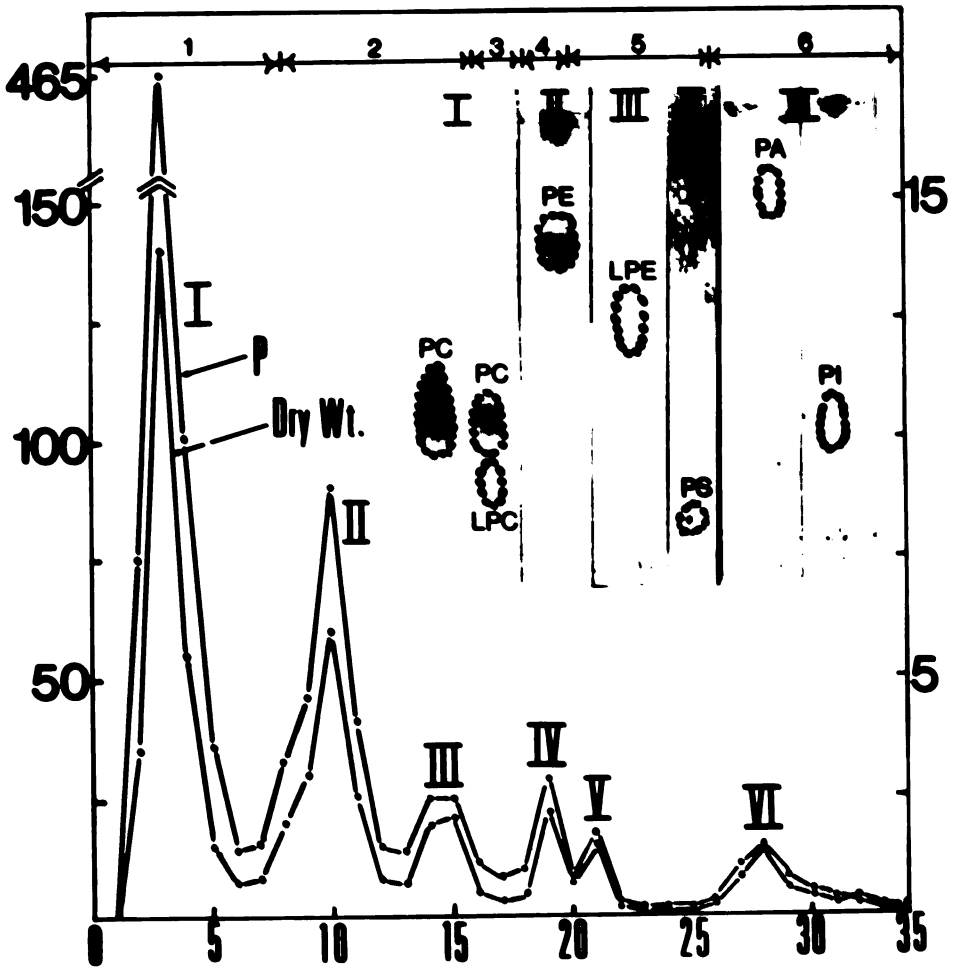
respectively, of the total lipid. The latter constituted 11% of the dry weight of the zoospore.

Phospholipid. The phospholipid was fractionated further on DEAE-cellulose, and the purity of each fraction was verified by TLC (Fig. 1). Peak I contained phosphatidylcholine (PC; R_f 0.26; all R_f values listed in this report are average values for many runs) and lysophosphatidylcholine (LPC; R_f 0.12). Both spots were molybdate positive and gave reactions for choline. Phosphatidylethanolamine (PE; R_f 0.72) and lysophosphatidylethanolamine (LPE; R_f 0.45) were found in peaks II and III, respectively. Both spots were ninhydrin and molybdate positive, as was phosphatidylserine (PS; R_f 0.00), the only compound in peak V. Peak IV was due to oxidation products of PE. Peak VI contained two components, phosphatidic acid (PA; R_f 0.78) and phosphatidylinositol (PI; R_f 0.25); both spots for peak VI were molybdate positive and ninhydrin negative. The quantitative composition of the total phospholipid is shown in Table 2.

The phospholipids were also characterized by determining their molar ratios of P:acyl esters:glycerol:N (Table 3). Theoretical and actual values agreed closely. The high acyl ester content of LPC was due to contamination with PC.

Glycerol phosphate esters obtained by deacylation of the phospholipids were examined by paper chromatography. Seven compounds were detected, each one reacting positively to the acid molybdate spray for P. The R_f values of the glycerylphosphoryl derivatives corresponded

Fig. 1. Representative elution patterns for milligrams dry weight of lipid (right axis) and micrograms of total P (left axis) obtained by column chromatography of total phospholipid on DEAE-cellulose. The column (2 by 20 cm) was loaded with 1,190 μg of phospholipid-P (obtained by silicic acid chromatography), and 50-ml fractions (horizontal axis) were collected at a rate of 3 ml/min; 1,163 μg of phospholipid-P (97.7%) were recovered. The elution sequence 1 to 6 (top) was as follows: chloroform-methanol (9:1, vol/vol), chloroform-methanol-acetic acid (7:3:0.002, vol/vol), methanol, chloroform-acetic acid (3:1, vol/vol), acetic acid, chloroform-methanol-ammonium hydroxide (32:8:1, vol/vol). The insert shows the results of TLC of compounds in peaks I to VI, phospholipid being visualized by H_2SO_4 charring. Both peaks I and VI contained two phospholipid components. Phosphatidyl choline (PC) and phosphatidic acid (PA) were in the peak fractions for I and VI, respectively, whereas lysophosphatidyl choline (LPC) and phosphatidyl inositol (PI) occurred in the corresponding shoulders.


1
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LPE
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25
30
35

TABLE 2. Composition of zoospore phospholipid

Component	Percentage[a]
Phosphatidylcholine	55.0 ± 3.0
Lysophosphatidylcholine[b]	6.3 ± 2.6
Phosphatidylethanolamine	22.1 ± 2.6
Lysophosphatidylethanolamine	6.3 ± 1.7
Phosphatidylserine	3.0 ± 1.0
Phosphatidic acid	3.0 ± 1.0
Phosphatidylinositol	4.3 ± 1.7

[a]Means and standard deviations for three experiments based on the amount recovered after column chromatography.

[b]Determination by P analysis after preparative TLC of column fractions containing lysophosphatidylcholine and phosphatidylcholine (see insert, Fig. 1).

TABLE 3. Molar ratios for zoospore phospholipids[a]

Compound	P:acyl esters:glycerol:N	
	Actual	Theoretical
PC	1.00:1.93:0.96:0.85	1:2:1:1
LPC	1.00:1.47:0.95:0.97	1:1:1:1
PE	1.00:2.13:1.09:0.97	1:2:1:1
LPE	1.00:1.08:1.00:1.13	1:1:1:1
PS	1.00:2.00:0.83:0.90	1:2:1:1
PA	1.00:1.96:1.01	1:2:1
PI	1.00:2.11:1.00	1:2:1

[a]The phospholipids used to establish molar ratios were taken either from column fractions (where purity was verified by TLC) or directly from TLC plates.

with published data (Kates, 1972) and those for our standards prepared from egg yolks. Two compounds (glycerylphosphorylserine and glyceryl-phosphorylethanolamine) were ninhydrin positive; two inositol-containing glycerylphosphoryl esters were detected.

Glycolipid. This lipid class was separated into five fractions on Florisil; seven different spots were derived therefrom by TLC (Fig. 2). Fraction I contained one component (R_f 0.86) which represented 10% of the total glycolipid as determined gravimetrically. Fractions II and III also contained one component each, both having an R_f of 0.55; they represented 38 and 32% of the total glycolipid, respectively. Another 12% of the glycolipid was present in fraction IV in the form of three components with R_f values of 0.31, 0.20, and 0.08. Fraction V had one component (R_f 0.88); it represented 8% of the glycolipid.

Results obtained with spray reagents suggested that all the foregoing substances were orcinol and diphenylamine positive except IVa and IVb; the latter were weakly orcinol and molybdate positive. The components in fractions II, III, and IVa were also ninhydrin positive.

The glycolipid fraction (15.8% of the total lipid [Table 1]) contained 6.8% of the P in the total lipid extract. Analyses by phenol-sulfuric acid and anthrone methods, with glucose as a standard, suggested that 23% of the glycolipid was carbohydrate. However, judging by enzymatic assays with glucose oxidase, only 6% of this carbohydrate was actually glucose.

Thin-layer and paper chromatography of the acid hydrolysis products indicated that, in addition to glycerol, several substances that behaved

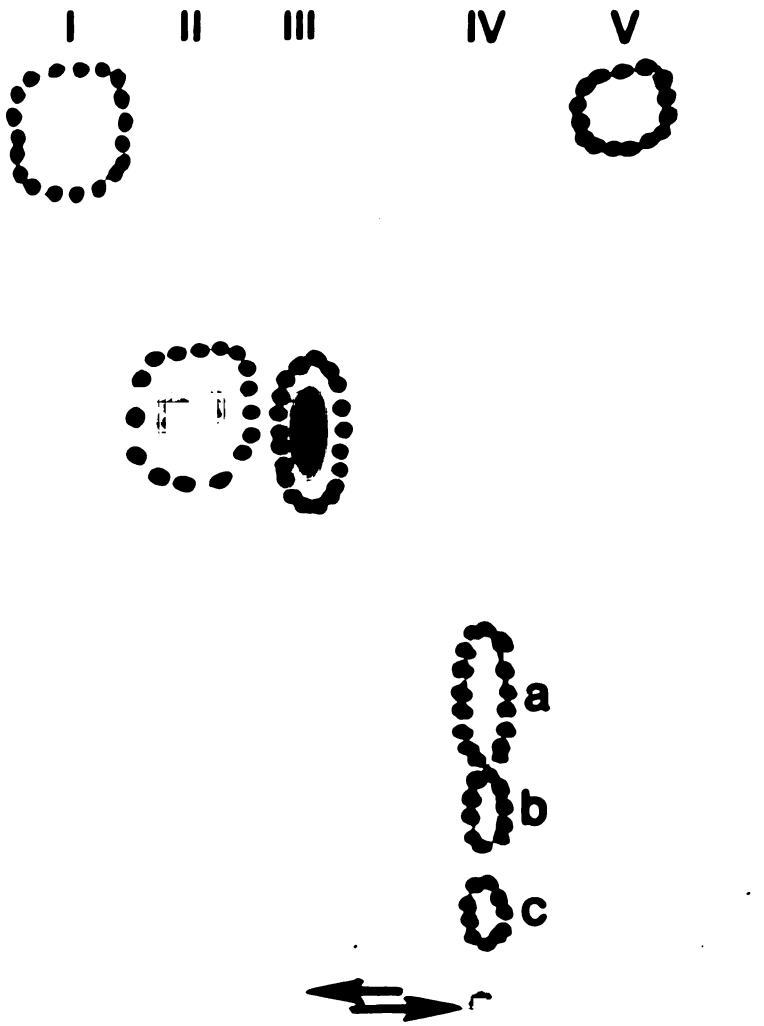


Fig. 2. Glycolipid fractions collected by Florisil column chromatography (1 by 15 cm) and resolved further by TLC. Fractions I to V were obtained by successive elutions with 50 ml of chloroform-acetone (1:1, vol/vol), acetone, 95% acetone, 90% acetone, and methanol. The chromatograms were visualized by H_2SO_4 charring.

like carbohydrates were present in the glycolipid. Fractions I and V appeared to contain monoglycosyldiglycerides judging from R_f values (Fig. 2) and the fact that each released only one carbohydrate after hydrolysis and TLC. Fractions II and III behaved like diglycosyldiglycerides chromatographically (Fig. 2), and each released two components, one of them being ninhydrin positive. Three carbohydrates were produced by Fraction IV, one of which was ninhydrin positive.

Neutral lipid. This group of lipids was resolved by TLC into 13 spots (Fig. 3). Neutral lipid was also column fractionated through two different media (Table 4), both yielding comparable results when assayed gravimetrically and monitored by TLC. The FFA, whether removed before fractionation on silicic acid or fractionated directly on Florisil, constituted 8% of the total neutral lipid. They migrated to or near the solvent front, as did the hydrocarbons, which accounted for another 13 to 14%. Triglycerides (TG; R_f 0.89) made up the major class (28 to 30%), whereas free sterols (FS; R_f 0.84) and sterol esters (SE; R_f 0.79 and 0.73) constituted 12 to 13% and 15 to 18%, respectively. Both FS and SE reacted positively to the $SbCl_3$ spray. Diglycerides (DG) represented 8 to 9% of the neutral lipid and contained four components (R_f 0.65, 0.58, 0.45, and 0.38), whereas monoglycerides (MG; R_f 0.30, 0.22, 0.09, and 0.00) represented 12%.

Fatty acid composition. Zoospores contain at least 20 fatty acids. The principal ones (Table 5) were palmitic, palmitoleic, stearic, oleic, γ-linolenic, and arachidonic acids. Nineteen fatty acids were

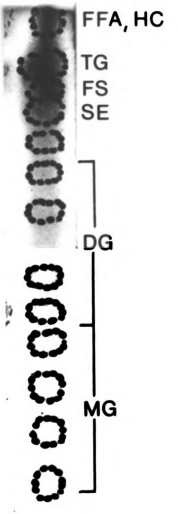


Fig. 3. Components in the neutral lipid fraction separated by TLC and visualized by H_2SO_4 charring. Neutral lipids are, from top to bottom: free fatty acids (FFA), hydrocarbons (HC), triglycerides (TG), free sterols, (FS), sterol esters (SE), diglycerides (DG), and monoglycerides (MG).

TABLE 4. Composition of zoospores neutral lipid[a]

Fraction	Percentage	
	By silicic acid	By Florisil
Hydrocarbons	14.0 ± 2.0[b]	13
Free fatty acids	8.3 ± 1.1	8
Triglycerides	29.8 ± 2.1	28
Free sterols	12.3 ± 1.7	13
Sterol esters	15.0 ± 1.0	18
Diglycerides	9.0 ± 1.4	8
Monoglycerides	11.6 ± 1.0	12

[a]Determined gravimetrically after fractionations on either silicic acid or 7% hydrated Florisil.

[b]Means and standard deviations based on three determinations.

TABLE 5. Principal fatty acids in zoospore lipid

Fatty acid[a]	Percentage of fatty acid in			
	Total lipid	Neutral lipid	Glyco-lipid	Phos-pholipid
16:0 (palmitic)	28	19	38	39
16:1 (palmitoleic)	4	4	5	3
18:0 (stearic)	9	9	3	2
18:1 (oleic)	32	35	23	14
18:3 (γ-linolenic)	12	6	18	23
20:4 (arachidonic)	7	5	0	13

[a]The first and second numbers represent length of carbon chain and number of double bonds, respectively.

detected in the neutral lipid fraction, palmitic and oleic acids accounting for over 50% of them. Eight fatty acids were found in the glycolipid fraction, palmitic, oleic, and γ-linolenic being the major ones, and arachidonic being conspicuously absent. The phospholipid fraction contained 12 fatty acids of which the major ones were palmitic, oleic, γ-linolenic, and arachidonic.

Lipid composition of sporangia and zoospores produced in liquid media. The proportions of neutral, glyco-, and phospholipid in zoospores derived from liquid cultures were very different than those for zoospores from agar media (Table 6, column 3 versus Table 1), even though the total lipid in the two kinds of zoospores was the same, i.e., about 11%. On the other hand, the proportions of these three lipid classes were about the same in the mature (i.e., post cleavage) zoospore-producing parent plants (column 2, Table 6) as they were in the zoospores themselves (column 3, Table 6); however, the lipid composition characteristic for both zoospores and postcleavage plants was different than that of precleavage plants (column 1, Table 6). These conclusions were substantiated by the distribution of radioactivity in the three lipid classes (columns 4, 5, and 6, Table 6) derived from precleavage plants, postcleavage plants, and zoospores from liquid cultures containing (^{14}C) acetate.

The three lipid classes derived from zoospores also resembled those from postcleavage plants when characterized further by TLC. The results obtained by similarly separating the labeled neutral, glyco-, and phospholipid via TLC and then scanning the chromatograms

TABLE 6. Composition of lipid in plants and zoospores produced in liquid media[a]

Fraction	Composition by weight (%)			Distribution of ^{14}C (%)		
	Pre-cleavage plants	Post-cleavage plants	Spores	Pre-cleavage plants	Post-cleavage plants	Spores
Neutral lipid	17.7	25.2	27.2	16.0	23.2	29.4
Glycolipid	13.1	12.0	12.4	15.3	11.7	11.6
Phospholipid	69.2	62.8	60.4	68.7	65.1	59.0

[a]Total lipid was extracted and separated as before (see Table 1) on silicic acid columns.

[b]Counted with a Tracerlab Versa/matic scaler.

for radioactivity are also delineated (Fig. 4). Most of the radioactivity in the neutral lipid fraction was associated with TG (A-I). FS (A-II) and SE (A-III) were also major components, whereas small amounts of label were associated with the MG and DG. Monoglycosyldiglyceride (B-I) and diglycosyldiglycerides (B-II) were the major components labeled in the glycolipid fractions; peaks B-III and B-IV are mixtures of glycolipids that were not resolved completely in this solvent system. PC (C-III) and PE (C-I) were the major labeled compounds in the phospholipid fraction, LPE (C-II) and LPC (C-IV) also being present. Minor phospholipids were PA, PI, and PS.

DISCUSSION

The wall-less, motile zoospores of B. emersonii possess an elaborate, tightly organized, membrane-bound corps of organelles; one especially prominent component is a cluster of discrete lipid globules partially wedged into a structure which, until its function and chemical composition has been at least partially characterized, is being called "SB matrix" (Truesdell and Cantino, 1971). These zoospores can develop along four different macrocylic pathways (Cantino, 1966), as well as a microcyclic pathway (Hennessy and Cantino, 1972), thereby producing plants which give rise to new generations of swarm cells. Against this background, we wish to comment briefly about the extractable lipid produced by this fungus.

Both zoospores and sporulating cells contain neutral lipids, glycolipids, and phospholipids, which vary in amount and relative proportions

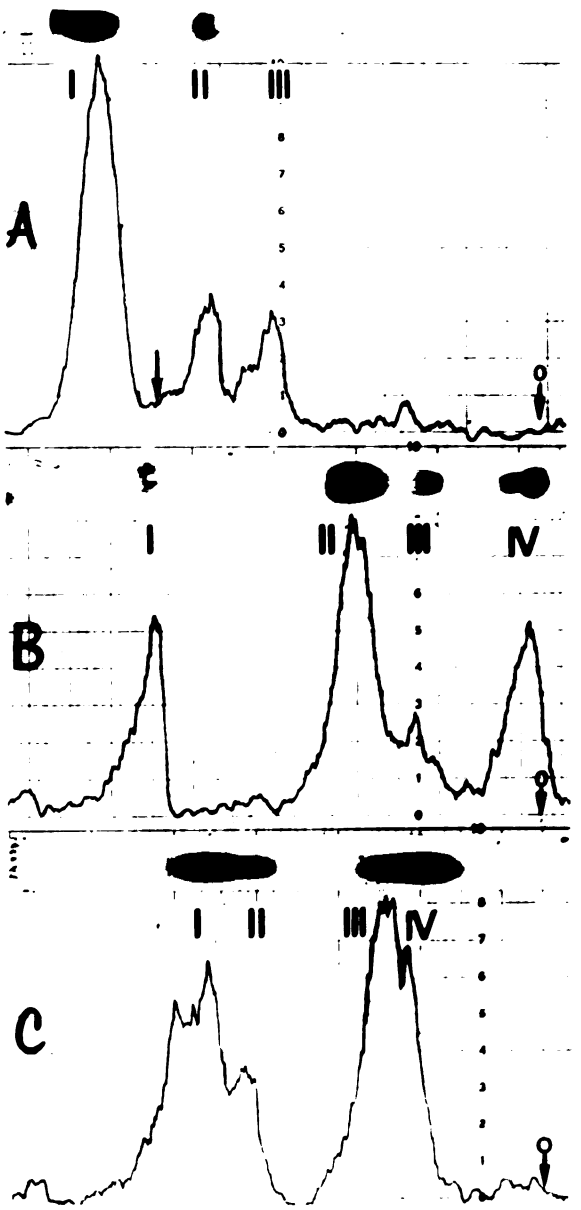


Fig. 4. Separable components in the ^{14}C-labeled neutral lipid (A), glycolipid (B), and phospholipid (C) extracted from postcleavage plants of B. emersonii and resolved by TLC, and the profiles for distribution of radioactivity in these components. The arrow (in A) indicates a shift to a 2.5-fold reduction in amplification of the signal; O represents the origin. Tracings were made with a Tracerlab 4-pi scanner.

depending on the developmental stage. Additionally, we have shown in this report that the proportions of these three lipid classes in zoospores derived from plate-grown cultures of ordinary colorless (OC) plants differed from the corresponding amounts in zoospores derived from liquid cultures; this observation is consistent with accounts showing that other microorganisms also change in lipid composition as they adjust to different environmental conditions (Bowman and Mumma, 1967; Kostiw et al., 1972). With B. emersonii, the temperature, pH, and composition of the media used for the two kinds of cultures were similar; however, population densities-especially when considered in relation to the amounts of oxygen apparently available-were quite different. We think this difference may account for the corresponding dissimilarities in lipid composition of the zoospores; our reasoning is as follows.

We observed (Table 6), as did Smith and Silverman (1973), that there was about 45 to 50% more neutral lipid in zoospores than in sporulating plants when both were derived from liquid cultures. The transition (precleavage plants → postcleavage plants → free swimming zoospores) was associated (Table 6) with a shift in neutral lipid from 16.8% through 24% to 28.3%, respectively. When grown in well-aerated liquid cultures, OC plants increase exponentially in weight up to about the time of sporogenesis (Goldstein and Cantino, 1962; Khouw and McCurdy, 1969); the corresponding increases in respiration and other evidence for oxidative activity (Khouw and McCurdy, 1969; McCurdy and Cantino, 1960), including the reutilization of lactic acid (Cantino, 1965), suggest

that there was a continuously increasing demand for O_2 up to the end of their generation time. In plate cultures, on the other hand, neither forced aeration nor agitation was employed; the demand for oxygen may have existed, but its availability was undoubtedly restricted by various diffusion processes and perhaps other factors; hence, the average O_2 uptake per plant was probably very much reduced, whereas lactic acid simultaneously accumulated in the medium. This information and the fact that the population density of the plants in plate cultures (ca. 2 x 10^5 thalli/ml after submersion in flooding water) was about five times greater than that in liquid cultures (ca. 0.4 x 10^5 plants/ml) lead us to suspect that exogenous oxygen deficiencies and corresponding shifts toward fermentative metabolism prior to and during sporogenesis may have been responsible, at least in part, for the increased proportion of neutral lipids in B. emersonii grown on solid media.

The composition of the extractable lipid in spores derived from plate cultures was also different than that in spores derived from liquid cultures. In the former, the major lipids were PC (20.3%), TG (13.5%), and diglycosyldiglycerides (DGDG; 13.3%), whereas PE, FS plus SE, and lysophosphatides (LP) accounted for 6.7, 12.2, and 4.9% of the lipid, respectively. In the latter, PC and PE were the most abundant components, and lesser amounts of TG, DGDG, LP, FS, and SE were also present, the phosphatides constituting 62% of the total lipid. These results are consistent with the phospholipid contents of zoospores derived from liquid cultures as reported by Smith and Silverman (1973) and Suberkropp and Cantino (1973), i.e., 55% and up to 85% of the

extractable lipid, respectively. The latter value is not out of line because it was unquestionably inflated by its inclusion of glycolipids; the fractionation techniques used at that time separated the total lipid into only two classes, neutral lipids and polar lipids.

Although the quantity of sterols and SE in the zoospores of B. emersonii seems to exceed the amount in most other Phycomycetes (Weete, 1973), the fatty acid composition of the B. emersonii zoospores does resemble that of other phycomycetes (Bowman and Mumma, 1967; Chenouda, 1970; Gordon et al. 1971; Shaw, 1966; Sumner and Morgan, 1969). In addition, γ-linolenic acid---once thought (Shaw, 1966) to be both characteristic of and limited to the phycomycetes, but recently found (Safe and Brewer, 1973) to occur in other fungi---was associated predominantly with polar lipids in the zoospores we analyzed, as it was in the mixture of variously aged thalli harvested from multiple generation of B. emersonii cultures by Sumner (1970). However, the average degree of saturation among the fatty acids we extracted from zoospores was greater (an approximate estimate can be derived from the data in Table 5) than the average value obtained by Sumner (1970) for plants. This apparent difference between spores and plants of B. emersonii could, of course, be due simply to differences in culture conditions rather than stages in ontogeny; on the other hand, our results do agree with Sumner's (1970) observations in that more polyunsaturated fatty acids were associated with polar lipids than with neutral lipids.

The glycolipid in B. emersonii seems to be of an unusual nature. It is also the most homeostatic of the three lipid classes, apparently

being stabilized at a level of about 12 to 15% of the total lipid whatever the developmental stage -- whether the glycolipid is derived from zoospores or sporangia, mature or immature, or extracted from plate-grown or liquid-grown cultures. This conclusion contrasts sharply with the results of Smith and Silverman (1973), who concluded that glycolipid accumulated during sporulation. Although they did not specify how many spores were released by their sporulating plants, the information provided suggests that they may have been microcyclic plants similar to the unispored plantlets studied by Hennessy and Cantino (1972). The physiological changes occurring after induction of sporogenesis in lag-phage (microcyclic) germlings and log-phase (macrocyclic) plants are known to show similarities but also some differences (Hennessy and Cantino, 1972). Perhaps the latter includes lipid composition.

Our unpublished data lead us to believe that the glycolipids are associated with specific organelles in B. emersonii, which could account for the stability of this class of lipids. A more detailed study is underway to identify the nature of these glycolipids and their possible relationships to certain functional aspects of the zoospore of B. emersonii.

II

Lipid Changes During Development of Blastocladiella emersonii

Fungi accumulate lipids (Cochrane, 1958) in the form of globules that probably serve as reserves (Walker and Throneberry, 1971). The zoospores and plants of the aquatic fungus Blastocladiella emersonii Cantino and Hyatt also contain lipid bodies (Cantino and Truesdell, 1970; Lessie and Lovett, 1968; Lovett and Cantino, 1960). Under conditions of starvation these zoospores utilize stored polysaccharides and lipid (Suberkropp and Cantino, 1973). Recently the lipid components have been identified and quantified (Mills and Cantio, 1974). The present report documents changes in lipid classes and their individual components during different development stages of B. emersonii.

MATERIALS AND METHODS

Culture techniques. Blastocladiella emersonii was grown at 22°C in 9 l. modified PGY broth (Myers and Cantino, 1971). The culture, inoculated with 2.5×10^8 - 4.5×10^8 spores obtained from PYG agar plates, was aerated (10 l./min) and illuminated with "cool white" fluorescent lighting, 555-712 $\mu W/cm^2$. After 9 h of growth 50 μCi of NaOAc-[U-^{14}C], (54 mCi/-mM) and NaOAc were added to give a final concn of 5×10^{-4} M. Growth was continued for an additional 9 h at which time aeration was stopped, the plants were allowed to settle, and the spent medium was removed by suction. The plants were washed once with 4 l. of sporulation inducing medium (0.5 mM MOPS [Calbiochem], pH 6.8, containing 0.1 mM $CaCl_2$) and resuspended in

1 l. of the same solution; aeration was resumed. From 10^{10} to 3.5 x 10^{10} spores were synchronously released 6-7 h later.

Suspensions of zoospores were passed through filter paper and concentrated by centrifugation (0 h spores) or they were treated in one of two ways: (a) the spores were allowed to swim in the aerated buffered medium for 5 to 10 h or (b) they were induced to encyst by chilling to 4-6°C (avg. time required to reach temp., 45 min). Then PYG broth was added immediately to full strength and equilibrated to 22°C. Approximately 95% encystment occurred within 15 min, this being faster than in most non-nutrient systems (Truesdell and Cantino, 1971). The cells were then extracted immediately or after 5 h of growth (germlings with mainly two nuclei).

Lipid extraction. Zoospores, cysts and germlings were concentrated by centrifugation (100 xg for 7 min), washed with H_2O and then recentrifuged. The pellets were suspended in 2:1 $CHCl_3$-MeOH (19 vol. solvent/vol. material), sonicated at 80 W for 30 sec, and extracted overnight at room temp. The extract was filtered through a coarse fritted glass Büchner funnel, evaporated to dryness under N_2, and suspended in 5 ml $CHCl_3$-MeOH (19:1). Non-lipid contaminants were removed with Sephadex G-25 (Rouser and Fleischer, 1965).

Chromatography. The lipid was then applied to a 2 x 8 cm silicic acid column and separated into classes. Neutral lipids were eluted with 100 ml $CHCl_3$, glycolipids with 100 ml Me_2CO and phospholipids with 100 ml MeOH. Each class was taken to dryness under a stream of N_2 at 40° and resuspended in 2 ml $CHCl_3$-MeOH (2:1). The fractions were then used for dry wt

determination, TLC, and the measurement of total ^{14}C incorporated. Neutral lipids were separated on Gelman ITLC-SG chromatography media, using light petrol-Et_2O-HOAc (80:20:1). Glycolipids and phospholipids were chromatographed on ITLC-SA and ITLC-SG media, respectively, using iso-PrOH-NH_4OH (100:7). Lipids were located by using H_2SO_4 and heat and identified by comparison with previously identified B. emersonii spore lipids (Mills and Cantino, 1974). Quantitative data for individual components were determined from total peak areas obtained from scans (Tracerlab 4 Pi scanner) for radioactivity in uncharred TLC's.

RESULTS

Radioactivity from NaOAc-[U-^{14}C] was incorporated into the lipids of synchronously produced zoospores (Fig. 5, 0 h). Following fractionation into neutral lipids, glycolipids and phospholipids, the percentage of each class was determined by both dry weight and total ^{14}C incorporated (Table 7, 0 h). Individual classes were separated by TLC (Fig. 6, 0 h) and the major lipid components were identified by techniques used earlier (Mills and Cantino, 1974) and quantified by measuring their total radioactivity (Fig. 6, 0 h). The TG and FS were the major neutral lipids, SE and MG being minor components with undectable label. The major glycolipid was DGDG, while the minor glycolipids were MGDG and PGDG. The phospholipid fraction contained two major peaks; one consisted of PC and LPC, the other contained PE and LPE. Minor phospholipids were PS and PI.

These data were then compared with the results obtained from zoospores which had been swimming for 5 and 10 h, from encysted spores, and from 5 h germlings.

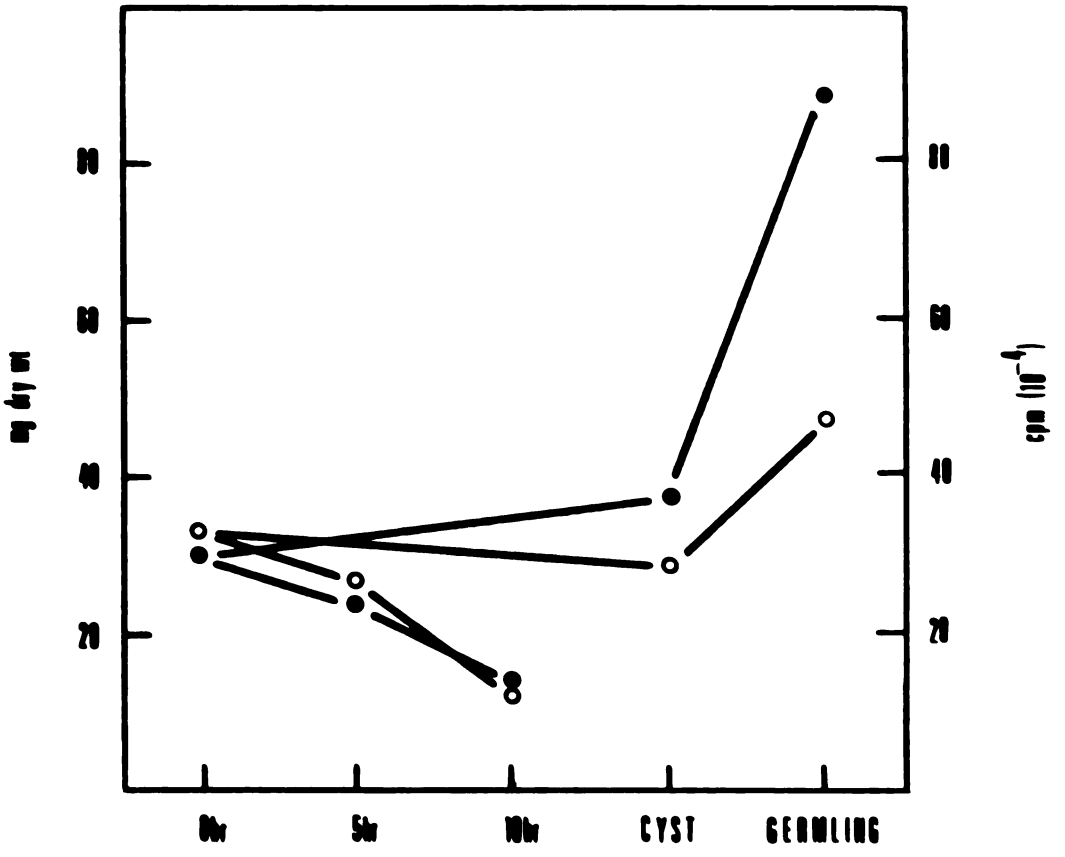


Fig. 5. Changes in total (●---●) and labeled (o---o) lipid/10^{10} cells during swimming and development. Lower curves, swimming spores; upper curves, encystment and growth.

TABLE 7. The percent composition of lipid during swimming and development as assessed by dry wt of the cells and incorporation of radioactivity (cpm) from NaOAc-[U-^{14}C].

	Neutral lipid		Glycolipid		Phospholipid	
Stage	Dry wt	cpm	Dry wt	cpm	Dry wt	cpm
0 h	19.9	21.3	17.8	17.2	62.3	61.5
5 h	15.9	11.6	18.6	15.1	65.5	73.3
10 h	25.8	22.6	24.2	24.7	50.0	52.7
Cyst	19.2	21.0	9.5	8.4	71.3	70.6
Germling	19.9	14.8	8.5	12.0	71.6	73.2

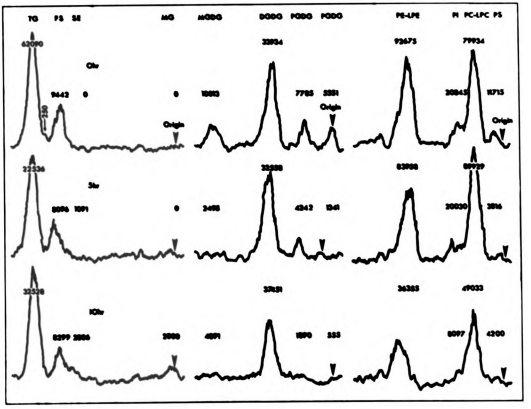


Fig. 6. Changes in profiles of labeled lipids in zoospores during swimming. For identity of peaks, see Abbreviations. Tabulated numbers are cpm incorporated in each peak 10^{10} spores. Full scale deflection was 100 cpm except where indicated (arrows).

Distribution of lipids in swimming zoospores. Zoospores were kept swimming in a non-nutrient buffered medium for either 5 or 10 h. Total lipid was then extracted and separated into classes and individual components as before. Total lipid, whether measured by dry weight or ^{14}C incorporation, decreased as spores were starved (Fig. 5; 0, 5 and 10 h). The per cent distributions for neutral lipids, glycolipids and phospholipids in the above were also compared (Table 7).

The individual lipid components within each class were examined (Fig. 6). The greatest change in the neutral lipid was in the TG; they decreased 64% after 5 h, then increased. Among the minor neutral lipids, SE and FS increased and decreased, respectively. Both complex glycolipids (PGDG) decreased after 5 and 10 h, while the MGDG decreased initially but then increased. The major glycolipid, a DGDG, also increased after 10 h. During the first 5 h the major phospholipids changed slightly, but after 10 h they decreased sharply. When compared to 0 h spores, the peaks containing PE and LPE, and PC and LPC, dropped by 61 and 39% respectively. PS and PI both decreased over the 10 h period.

Distribution of lipid in encysted spores and in germlings. Lipid was extracted from zoospores which had been induced to encyst and from germlings which had been grown in a nutrient medium. As before, the lipid was separated first into classes and then into individual components. The sp. act., as expected, decreased since no additional ^{14}C had been added (Fig. 5; cysts and germlings). However, the percent composition (Table 7) for each of the classes, as determined by total ^{14}C incorporated, was in

reasonable agreement with that calculated from their respective dry weights. When 0 h spores were compared to cysts and germlings (Table 7) it was found that the neutral lipids remained constant, the glycolipids decreased, and the phospholipids increased.

Changes occurring in the major lipid components can be followed in Fig. 7. With respect to neutral lipids, it is evident that the labeled TG decreased, while the SE increased. On the other hand, the FS dropped during encystment but then increased upon subsequent growth. One particularly conspicuous change in the neutral lipids of germlings was the appearance of the previously non-detectable MG. As for glycolipids, 55% of the DGDG and essentially all of one of the two complex PGDG were utilized upon encystment. Both of these components, however, regained almost their original levels after 5 h of growth. The other PGDG and the MGDG decreased by about half upon encystment but increased greatly in the growth phase. Finally, the phospholipids PS, PI, and the peak containing PE and LPE decreased upon encystment whereas PC and LPC increased. All labeled phospholipids increased after 5 h of growth, with PE and LPE becoming the major constituents.

DISCUSSION

Zoospores of B. emersonii can swim in nutrient-free media for extended periods of time (Suberkropp and Cantino, 1973; Soll and Sonneborn, 1972) during which lipid is utilized (Suberkropp and Cantino, 1973). Supportive electron micrographs (Suberkropp and Cantino, 1973) of starving zoospores showed an apparent progressive decrease in the size and number

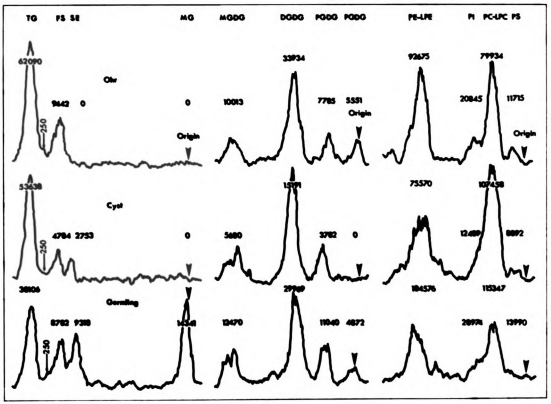


Fig. 7. Changes in profiles of labeled lipids in zoospores, cysts and germlings.

of lipid bodies as well as a continuous decrease in the size of the "lipid sac matrix" (also called "SB-matrix" or simply "SB"; Cantino and Truesdell, 1970), an organelle of unknown function. Our preliminary studies of isolated SB-lipid complexes suggest that the SB matrix contains a large amount of MGDG. Therefore, since MGDG is partially depleted during swimming, its loss might have been responsible for the decrease in the size of the SB-matrix. The major lipid component utilized during 5 hr of swimming, however, is the TG; we suggest, therefore, that this is the major lipid constituent of the lipid globules.

After 5 h of swimming the TG had leveled off; between 5 and 10 h, it increased while phospholipids decreased sharply. The utilization of phospholipid as energy sources has been cited for zoospores of Phytophthora capsici (Gay et al., 1971), mammalian spermatozoa (Bishop, 1962; Higashi and Kawai, 1968), and Tetrahymena pyriformis (Levy and Elliott, 1968). Interestingly, there was a transitory increase in cytoplasmic triglyceride globules during starvation of Tetrahymena; the authors felt that fatty acids released by catabolism of phospholipid, possibly coming from broken mitochondria, were temporarily stored in the form of triglycerides.

During encystment of B. emersonii, major changes take place in the glycolipids. All glycolipid components decrease; one, a PGDG, is completely utilized. The main glycolipid, a DGDG, decreases by 55%. This glycolipid is a major lipid constituent of the gamma particle (Myers and Cantino, 1974), an organelle involved in cyst wall synthesis (Cantino and Myers, 1972; Truesdell and Cantino, 1971). The neutral lipid class remains constant upon encystment, while the phospholipid increases, due mainly to PC and LPC.

After 5 hr of growth, glycolipids had exceeded their level in 0 hr spores, while the phospholipid increased, due primarily to LPE and PE. The neutral lipids also changed conspicuously during growth with both the SE and MG being synthesized. The MG are probably being used for the synthesis of more complex neutral lipids and possibly indirectly for the synthesis of glycolipid and phospholipid components (Jack, 1965). The reason for the increase in SE is not known although similar increases have been reported to occur during sorocarp formation of Dictyostelium discoideum (Long and Coe, 1974). Answers to this and related questions will hopefully be forthcoming from current studies of changes in the lipid content of subcellular organelles during development of B. emersonii zoospores.

III

Isolation and Characterization of Lipid Globules from the Zoospores of *Blastocladiella emersonii*

Lipid globules have been identified in zoospores of *Blastocladiella emersonii* (Cantino and Truesdell, 1970; Myers and Cantino, 1974) and in other fungi (Weber and Hess, 1976) by their staining qualities, morphology and osmiophilic properties; however, little is known about their chemistry. The lipids of *B. emersonii* zoospores have been characterized quantitatively (Mills and Cantino, 1974) and their changes during different developmental stages have been determined (Mills et al., 1974). The zoospore also contains a microbody of unusual size (Mills and Cantino, 1975a) in close proximity with the lipid globules and the spore's single mitochondrion. This microbody contains the glyoxylate cycle enzymes malate synthase and isocitrate lyase (Mills and Cantino, 1975b). As zoospores swim there is an increase in activity of these glyoxylate cycle enzymes (Mills and Cantino, 1975b). There is also a decrease in lipid triglyceride (Mills et al., 1974) and the lipid globules become increasingly hard to find when swimming zoospores are examined ultrastructurally (Suberkropp and Cantino, 1973). It has, therefore, become desirable to correlate the above events by further characterization of the lipid globules. This paper describes the isolation and chemical analysis of the lipid globules from *B. emersonii* zoospores.

MATERIALS AND METHODS

Culture conditions. B. emersonii (Cantino and Hyatt, 1953) was grown on Difco PYG agar by inoculating standard Petri dishes with 4 x 10^5 spores per plate and culturing at 22°C in the dark. After 24 h each plate was flooded with 5 ml H_2O and the zoospores were collected 15 min later by filtration. Population densities were established with a model B Coulter counter and the zoospores were sedimented at 1000 x g for 7 min. The yield was 3 x 0^9 - 4 x 10^9 zoospores per 100 plates.

Isolation of lipid globules. Zoospore pellets were suspended in 10 ml of 50 mM Tricine buffer, pH 7.5, containing 1 mM EDTA. The zoospore suspension was frozen, thawed and sonicated (30 sec, 50 watts, 4°C) until lipids were free floating as determined microscopically. The spore homogenate was centrifuged for 30 min at 4°C and 20,000 rpm (40,000 x g - 70,000 x g) in a swinging bucket rotor #969, model BD-2 International ultracentrifuge. The aqueous content of the centrifuge tube was frozen in a dry ice-ethanol bath and the lipid layer at the top of the tube was carefully suspended in a few milliliters of the Tricine buffer. The suspended lipids were mixed with 3 ml of 30% (w/v) sucrose which was layered below 7 ml of Tricine buffer. The crude lipid preparation was purified by floatation through the Tricine-sucrose medium by centrifugation at 20,000 rpm for 30 min at 4°C. The floating lipid layer was collected after freezing of the tube contents (as described above), and the washing procedure was repeated once. The lipids were collected by carefully puncturing the tube and allowing the infranatant to drain, thus leaving the lipids adhering to the sides of the centrifuge tube.

Lipid and protein extraction. The lipids were washed from the sides of the tube with 20 volumes of $CHCl_3$:CH_3OH (2:1, v/v). The lipid extract was mixed with 0.2 volume of 3.6 mM $CaCl_2$ and the emulsion was centrifuged for phase separation. The top layer was saved for protein determination, while the bottom layer was filtered through a coarse fritted-glass Büchner funnel. The filtrate was evaporated to dryness under N_2 and the residue was dissolved in 5 ml $CHCl_3$:CH_3OH (19:1, v/v). Remaining non-lipid contaminants were removed with Sephadex G-25 (Rouser and Fleischer, 1965).

Chromatography. The lipid was applied to a silicic acid column (10 g, 100 mesh silicic acid; 2 x 8 cm column) and separated into classes. Neutral lipids were eluted with 100 ml of $CHCl_3$ while phospholipids were eluted with 100 ml CH_3OH. Each class was reduced to dryness under a stream of N_2 at 40°C and dissolved in 1 ml of $CHCl_3$:CH_3OH (2:1, v/v). Neutral lipids were separated on Gelman ITLC-SG chromatography media using petroleum ether-diethyl ether-acetic acid (80:20:1 v/v); phospholipids were chromatographed on the same media using isopropanol-ammonium hydroxide (100:7, v/v). Lipids were detected by using H_2SO_4 and heat and their identities determined by comparison with previously identified B. emersonii spore lipids (Mills and Cantino, 1974). Neutral lipids were separated further on silicic acid columns after removal of free fatty acids (Dittmer and Wells, 1969). The purity of each fraction was checked by thin-layer chromatography for neutral lipids as described above.

Analytical procedures. Lipid-P was determined after digestion of samples with 10 N H_2SO_4 by a modification of Bartlett's method (Bartlett, 1959). Phospholipid was calculated from values of total lipid-P assuming a 4% P content in phospholipids. Total and free sterols were estimated by sterol analysis (Kates, 1972) of fractions eluted after silicic acid chromatography of the neutral lipid. Total and neutral lipids were determined gravimetrically. Protein was determined by the Lowry et al. method (1951), using bovine albumin Fraction V as a standard.

Carotenoid analysis. The isolated lipids were dissolved in $CHCl_3$:CH_3OH (2:1 v/v) and reduced to dryness with N_2. They were dissolved in petroleum ether, again reduced to dryness with N_2 and dissolved in light petroleum ether (b.p. 30°C-60°C). Spectra were established in light petroleum ether, chloroform and carbon disulfide, and absorption peaks were compared with those found in the literature (Goodwin, 1952, 1955). Carotenoids were separated by thin-layer chromatography on activated (100°C for 30 min) Eastman alumina (#6063) and Eastman Silica gel (#6060) chromogram sheets using hexane-benzene (90:10 v/v). Quantitative determination was based on $E^{1\%}_{1cm}$ values for γ-carotene (Goodwin, 1955).

Electron microscopy. The isolated lipid globules were fixed with a 1:1 mixture of 1% glutaraldehyde and 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at 4°C. The lipid globules were pelleted at 500 x g for 5 min and rinsed three times with cacodylate

buffer. They were then post fixed 1 h at 4°C with 2% OsO_4, pelleted and rinsed as above. They were dehydrated through a graded series of alcohol followed by propylene oxide, and embedded in Spurr's low viscosity resin (Spurr, 1969). Thin sections were examined with a Phillips 300 electron microscope.

RESULTS

Morphology of lipid globules. The zoospores of B. emersonii contain about 9 lipid globules per spore (based on counts of 168 zoospores; mean = 9.1; st. dev. = 1.8); they are approximately 0.3 - 0.6 μm in diameter and roughly spherical in shape. These lipid globules are usually separate, but groups of 2 - 3 are sometimes found in thin sections. The in situ appearance and position of the lipid globules in the zoospore is presented in Fig. 8a. An electron micrograph of the isolated floating lipid layer is shown in Fig. 8b. The diameters of most of the isolated lipid globules range from 0.4 - 1.5 μm, although some larger globules are present. These probably represent lipids which have coalesced during isolation. Some non-lipid contaminants, probably membrane fragments, are also present. In keeping with earlier conclusions (Cantino and Truesdell, 1970), there is a conspicuous lack of any obvious membrane surrounding the globules, although a thin layer of denser material can sometimes be seen surrounding them; however, this layer does not resemble a trilaminar membrane (Fig. 8c).

Composition of the lipid globules. Analyses of the floating lipid layer are presented in Table 8. All the data are given in terms of pg/spore

Fig. 8. (a) Electron micrograph of a thin section through B. emersonii zoospore showing the in situ appearance of the lipid globules (L) and their relationship to the spore's single mitochondrion (M) and the symphyomicrobody (SB). (b) Thin section of the isolated lipid layer. (c) High magnification of lipid globule. Calibration bar = 0.5 μm for a and b, 0.1 μm for c.

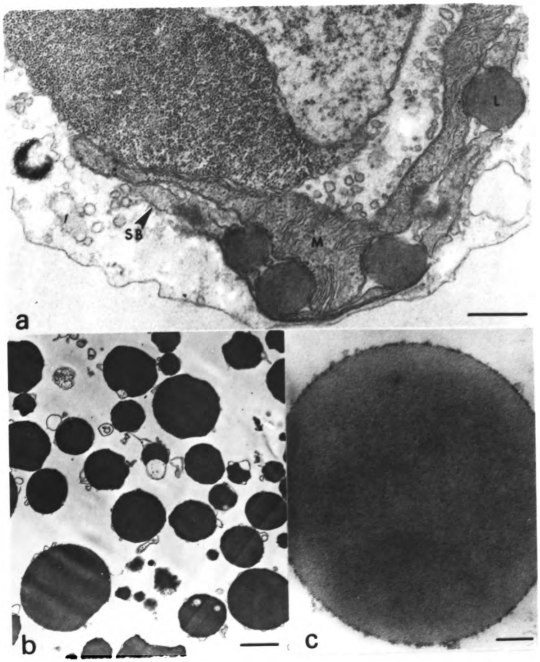

L
SB
M
a
b
c

Table 8 Composition of Lipid Globules

Composition is expressed as pg/spore. Values represent the means ± standard deviations. Numbers in parentheses correspond to the number of experiments.

Protein	.034 ± .006 (8)
Total lipid	.742 ± .045 (6)
Neutral lipid	.722 ± .043 (6)
Phospholipid	.019 ± .004 (8)

since it was possible to obtain accurate spore counts with the Coulter counter. Both protein and phospholipid were consistently associated with the lipid layer, although their presence might have been due to the contaminating membrane fragments. The protein and phospholipid accounted for 4.4% and 2.5%, respectively, of the total dry weight of the lipid layer. Two phospholipids were present in the phospholipid fraction after silicic acid chromatography. Phosphatidylcholine was the major phospholipid, phosphatidylethanolamine the minor one. These are the major phospholipid components of the zoospores of B. emersonii (Mills and Cantino, 1974). No glycolipids were found. The sum of protein and lipid for six experiments was 88-99% of the dry weight of the total floating lipid layer. The neutral lipids were the major components, representing 93.2% of total weight of the lipid globules.

When the neutral lipid fraction was separated by silicic acid chromatography (Table 9) the main lipid component was triglyceride. It accounted for 84.5% of the total weight of the lipid globules. The triglyceride associated with the latter represented 89% of the total zoospore triglyceride. Free sterols accounted for 7.4% of the dry weight of the lipid globules. Free sterols reacted positively to the $SbCl_3$ spray (Skipski and Barclay, 1969) after separation by thin-layer chromatography. The free sterols associated with the lipid globules represented 26% of the total zoospore free sterols. Diglycerides and free fatty acids were found in trace amounts (<1%), while no hydrocarbons, sterol esters or monoglycerides could be detected.

The floating lipid layer had a yellow color, and its absorption spectrum in light petroleum ether yielded three peaks with maxima at

TABLE 9. Composition of the separated neutral lipid associated with the lipid globules after silicic acid column chromatography[a,b,c]

Triglyceride	.647 ± .006 (3)
Free sterol	.057 ± .021 (3)
Diglyceride	Trace
Free fatty acid	Trace
Sterol esters	Not detected
Monoglycerides	Not detected

[a]Data are expressed as the means ± standard deviations. The number in parentheses correspond to the number of experiments. Data are expressed as pg/spore.

[b]TG determined gravimetrically; FS and SE determined as outlined in Materials and Methods.

[c]Trace amounts were <1%.

490, 458 and 433 nm. These absorption maxima are very close to those of the γ-carotene found in Phycomyces blakesleeanus (Goodwin, 1952). The absorption peaks were shifted to 507, 474 and 447 nm in $CHCl_3$ and to 528, 495 and 467 nm in CS_2. These values are also in reasonable agreement with those reported for γ-carotene (Goodwin, 1955). Only one carotenoid was detected by thin layer chromatography. Based on the $E^{1\%}_{1cm}$ value of 2720 for the peak maximum for γ-carotene in light petroleum ether (Goodwin, 1955), this caretenoid represented 0.04% of the total weight of the lipid globules.

DISCUSSION

The lipid globules isolated from B. emersonii are compared with lipids isolated from other organisms in Table 10. There seem to be two classes of lipid particles. One class is small in size and high in protein. Representatives of this class are liposomes from liver (Schlunk and Lombardi, 1967) and composite lipid vesicles from cotyledons of bush beans (Allen et al., 1971; Mollenhauer and Totten, 1971). All the other examples in Table 3 are from the other class of isolated lipid which is larger in size and low in protein. The lipid globules of B. emersonii are in this class. All the isolated lipids are spherical in shape and may or may not have a surrounding membrane. These lipids are thought to function in energy storage and/or membrane synthesis (Borowitz and Blum, 1976; Clausen et al., 1974; DiAugustine et al., 1973).

We believe that the lipid globules in the zoospores of B. emersonii function primarily as a storage region for fatty acids in the form of triglyceride. These are used in energy production since the metabolic

activities of the zoospores are catabolic, not anabolic (Cantino and Mills, 1976; Lovett, 1975; Soll and Sonneborn, 1971; Suberkropp and Cantino, 1973). Our reasoning is as follows. The zoospore contains a side body complex (Cantino and Truesdell, 1970), this being a tight association of lipid globules, a single mitochondrion and a symphyo-microbody (Mills and Cantino, 1975a). If the zoospores are permitted to swim in a non-nutrient medium (starvation conditions), the lipid globules become fewer in number and smaller in size when viewed ultrastructurally (Suberkropp and Cantino, 1973). This observation is supported by chemical evidence. There is a 64% loss in triglyceride as the zoospores swim for 5 h (Mills et al. 1974). There is also a three-fold increase in the activities of glyoxylate cycle enzymes after 5 h (Mills and Cantino, 1975b). The association of lipid globules in intimate contact with a microbody on one side and a mitochondrion on the other forms an ideal arrangement for the metabolism of the lipid by a glyoxysomal-type particle.

Although the pigmented lipid globules in the male gametes of Allomyces, a close relative of B. emersonii, are thought (Emerson and Fox, 1940; Turian, 1969) to carry γ-carotene, direct evidence that carotenoids are associated with isolated lipid particles is rare (Johnston and Hudson, 1976). The function of the carotenoid in the lipid globules of B. emersonii is unknown.

TABLE 10. Comparison of isolated lipid globules from B. emersonii with other isolated lipids

Source & Ref.	Name	Size (μm)	Membrane	% Composition TG	DG	MG	FS	SE	FFA	PL	Protein
B. emersonii	Lipid globule	0.4-1.5	none	84.5	trace[a]	none	7.4	none	trace	2.5	4.4
rat liver DiAugustine et al., 1973	Lipid droplet	0.5-2.0	present	86.1	trace	none	trace	8.6	none	1.7	2.8
Saccharomyces cerevisiae Clausen et al., 1974	Lipid particle	0.3-1.0	none	47	-	-	-	44	0.5	0.5	6.0
Castor bean seeds Harwood et al., 1971	Oil droplet	0.1-3.5	none	70	-	-	-	-	-	-	-

Table 10 (cont'd.)

Rabbit liver Mackenzie et al., 1966	Cytoplasmic lipid-rich particle	0.5-1.5	present	90	2.4	0.9	trace	1.6	-	2.0	3.0
Peanut Cotyledons Jacks et al., 1967	Spherosome	1.0-2.0	present	97.9[b]	-	-	-	-	-	0.8	1.3
Rat liver Bar-on et al., 1971	Floating triglyceride	0.5-2.0	-	93[c]	-	-	-	-	1.9	1.7	-
Rat liver Schlunk & Lombardi, 1967	Liposomes	0.15-0.25	present	35.2	1.1	0.4	1.0	0.3	2.2	22.7	37.1

Table 10 (cont'd.)

<u>Lipomyces starkeyi</u> Uzuka et al., 1975	Oil globule	1.0-2.0	present	86[d]	5.7	-	-	1.0	4.7	3.0	+[e]
Bush bean cotyledons Allen et al., 1971 Mollenhauer & Totten, 1971	Simple lipid vesicles	0.5-3.0	present	92.4[f]	-	-	-	-	-	2.3	5.3
Bush bean cotyledons Allen et al., 1971 Mollenhauer & Totten, 1971	Composite lipid vesicles	0.1	present	64.9[f]	-	-	-	-	-	7.2	27.9

Table 10 (cont'd.)

[a]present but no values given.

[b]reported as total lipid, but mainly triglyceride.

[c]TG and DG fractions were combined

[d]Based on distribution of radioactivity.

[e]Protein was present and equivalent to 10 μg/ml of lipid layer.

[f]Calculated from ratios given assuming 1 gm protein and an average molecular weight of phospholipid as 684.

IV

The Single Microbody in the Zoospore of Blastocladiella emersonii is a 'Symphyomicrobody'

'Microbodies' which have been found in most major groups of organisms (DeDuve, 1969), display some or all of the following features (Mollenhauer et al., 1966; Frederick et al., 1968; Hruban and Rechcigl, 1969): a round to elongate structure 0.3 μ to 1.5 μ in diameter and delimited by a ca. 60 Å - 80 Å membrane; a close association with endoplasmic reticulum or lipid bodies; a matrix of moderate electron density in which electron opaque inclusions; crystalloid structures, or both may be embedded. Microbodies have also been categorized physiologically as either peroxisomes or glyoxysomes (DeDuve, 1969; Tolbert, 1971). Peroxisomes play a role in glycolate metabolism and photorespiration, and produce and degrade H_2O_2 (DeDuve, 1969; Tolbert, 1971). Glyoxysomes, frequently associated with lipid bodies (Vigil, 1970, 1973; Mendgen, 1973; Silverberg and Sawa, 1973; Trelease et al., 1974), are involved in converting fats to carbohydrates (Beevers, 1969; Tolbert, 1971).

In recent years, workers have labeled various particles in the zoospores and sporangia of aquatic fungi as microbodies. However, none of them has published evidence for the origin, fate, or enzyme content of such organelles, nor any other significant supporting evidence that they are truly microbodies. In this report, we provide ultracytochemical evidence for the occurrence of microbodies in zoospores and sporangia of the fungus Blastocladiella emersonii Cantino and Hyatt. We also present arguments for the method of origin of a novel microbody, here named

the 'symphyomicrobody', and discuss its possible function in the zoospore and its fate after encystment.

MATERIALS AND METHODS

B. emersonii was cultured at 22°C in the dark on Difco PYG agar in standard Petri plates after inoculation with ca. 4 x 10^5 spores. The subsequent first generation of zoospores was pre-fixed on the plates for 10 min with 0.5% glutaraldehyde (GTA) in 0.1 M sodium cacodylate (SC) at pH 7.2, filtered, centrifuged (500 x g, 5 min), and fixed (2 h, 4°C) with 2% GTA in SC. Zoosporangial plants were grown at 22°C in PYG broth (Myers and Cantino, 1971), collected (500 xg, 3 min) at the time of papilla formation (ca. 22 h after inoculation), and fixed (1 h, 22°C) with 2% GTA in SC. Zoospores and sporangia were rinsed thrice with SC, pre-incubated in 0.05 M 2-amino-2-ethyl-1,3 propanediol buffer (AEP), pH 10, for 30 min, and then incubated at 37°C for 60 and 90 min, respectively, in the complete reaction mixture: 5 ml 0.05 M AEP, 0.1 ml 3% H_2O_2, and 10 mg 3,3'-diaminobenzidine 4 HCl (DAB). The final pH was adjusted to 9. Cells were also incubated in complete reaction mixtures containing 0.01 M KCN, and in control mixtures without H_2O_2. After incubation, cells were rinsed for 30 min in 0.05 M AEP (pH 9), rinsed thrice in SC, postfixed in 2% OsO_4 (1 and 2 h, respectively; 22°C), again rinsed thrice in SC, dehydrated through a graded series of alcohol followed by propylene oxide, and then embedded in Spurr's low viscosity resin (Spurr, 1969). Thin sections were examined with a Philips 300 electron microscope.

RESULTS AND DISCUSSION

The posteriorly uniflagellated zoospores of B. emersonii contain a

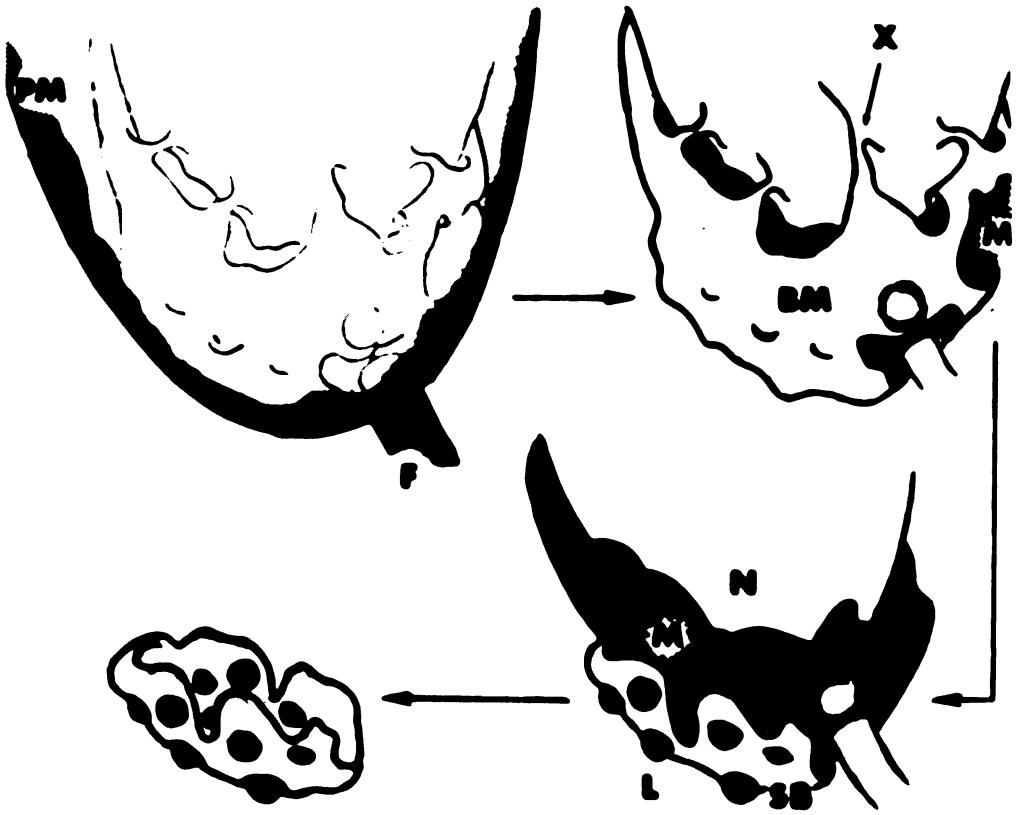
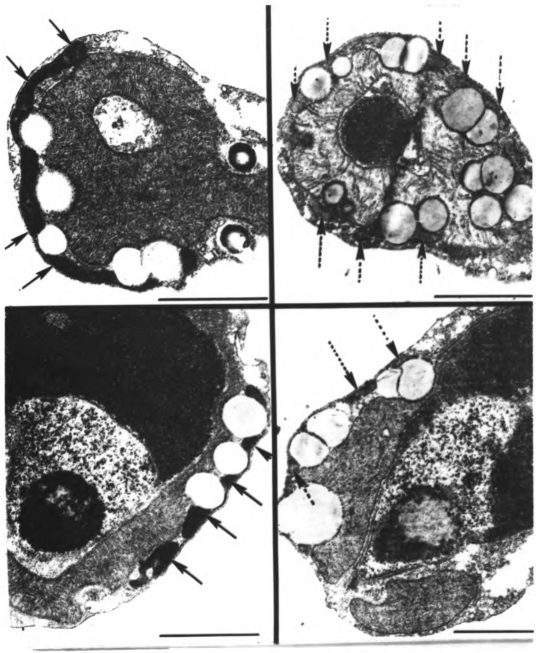


Fig. 9. The relationship of the symphyomicrobody---lipid complex to other organelles in the side body of the Blastocladiella emersonii zoospore. Lipid granules (L) are molded against the symphyomicrobody (SB), a spoon-bowl shaped organelle with an irregularly wavy rim. The SB lies next to the long 'arm' of the mitochondrion (M), usually in intimate contact with it. A sheet of double membrane (the backing membrane; BM) covers the SB-L-M complex, and is 'attached' at several places (e.g., as at X), i.e., is continuous with, the outer unit membrane around the nucleus and its nuclear cap. All of this is surrounded, in turn, by the plasma membrane (PM) of the zoospore. The single flagellum is also shown.

nucleus, a nuclear cap, gamma particles, and a side body complex (Cantino et al., 1963; Cantino and Truesdell, 1970); the latter consists of a single mitochondrion, lipid granules, and the SB matrix (Fig. 9). The SB matrix resembles a microbody in some respects; it is unit membrane limited, somewhat electron opaque, and always closely associated with lipid bodies---a common characteristic of glyoxysomes. But, in comparison with a typical microbody, the SB matrix is gigantic (although this could conceivably be misleading in that its size is based on a three dimensional model derived from studies of serial sections [Cantino and Truesdell, 1970] whereas that of most other microbodies has been based on two dimensional measurements of random thin sections). Another difference stems from the fact that microbodies are frequently associated with endoplasmic reticulum (Vigil, 1973), while the zoospores of B. emersonii do not possess an endoplasmic reticulum (Cantino et al., 1963).

Microbodies have been identified by the use of ultracytochemical techniques. Although many enzymatic activities have been used as 'markers' for microbodies, only catalase apparently occurs in almost all of them (DeDuve, 1969). It can be demonstrated ultracytochemically (Novikoff and Goldfischer, 1968; Frederick and Newcomb, 1969), and its presence has been used to establish the presence of microbodies in various organisms (Beard and Novikoff, 1969; Frederick and Newcomb, 1969; Vigil, 1970; Matsushima, 1972; Stewart et al., 1972). We have applied the procedure of Beard and Novikoff (1969) to B emersonii zoospores. Incubation in the DAB/H_2O_2 medium produced a very dense deposit over the SB matrix (Fig. 10), but not elsewhere. The reaction did not occur when H_2O_2 was absent, and it was partially inhibited by 0.01 M KCN. It appears,

Fig. 10. The response of the SB matrix in zoospores of B. emersonii to the Beard and Novikoff DAB test for catalase activity. The reaction product is localized in the SB matrix (solid arrows, top and bottom left) of the test spores, but not in the control spores (broken arrows, top and bottom right). Bar = 1.0 μ.



therefore, as if the SB matrix contains catalase, and that it may be a sort of microbody.

If the SB matrix is a microbody, what is its origin? The genesis of microbodies has been associated with endoplasmic reticulum (Matsushima, 1972; Vigil, 1973; Silverberg and Sawa, 1973; Richardson, 1974). The zoospores of B. emersonii do not have endoplasmic reticulum; it is present, however, in developing sporangia, and therein it is closely associated with numerous, small, irregularly shaped, single membrane bound particles of moderate electron density labeled 'sb granules' (Lessie and Lovett, 1968). When we tested B. emersonii sporangia for catalase ultracytochemically in an attempt to locate possible precursors of the SB matrix, only the sb granules gave a positive reaction (Fig. 11). Lessie and Lovett (1968) showed that these granules, along with lipid bodies, surround the nucleus during sporogenesis, and that both sb and lipid then become aligned alongside the mitochondrion in the finished zoospore while they are still inside the sporangium. Since it has also been shown (Cantino and Truesdell, 1970) that, in free swimming zoospores, such sb granules are interconnected into a single, large, unit membrane bound SB matrix (Fig. 9), it is now reasonable to conclude that the SB matrix is formed by 'symphyogenesis'---i.e., that it is produced by the union of formerly separate elements (Fig. 12). And, since we herewith provide evidence that the SB matrix possesses catalase activity, and that it is the only organelle in the zoospore that does so, the SB matrix can properly be called a 'symphyomicrbody'. The building blocks from

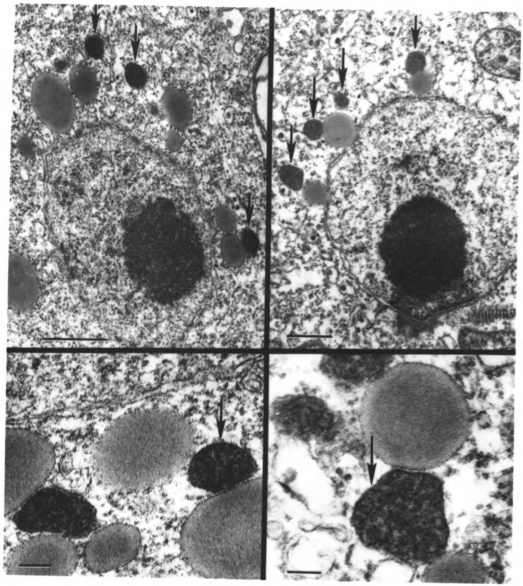


Fig. 11. Sections through sporangia at sporogenesis (ca. 22 h. after inoculation). Arrows point to the 'sb granules'. Catalase positive reactions are seen at top and bottom left, and negative reactions in controls at right top and bottom. Bars = 0.5 μ and 0.1 μ, top and bottom respectively.

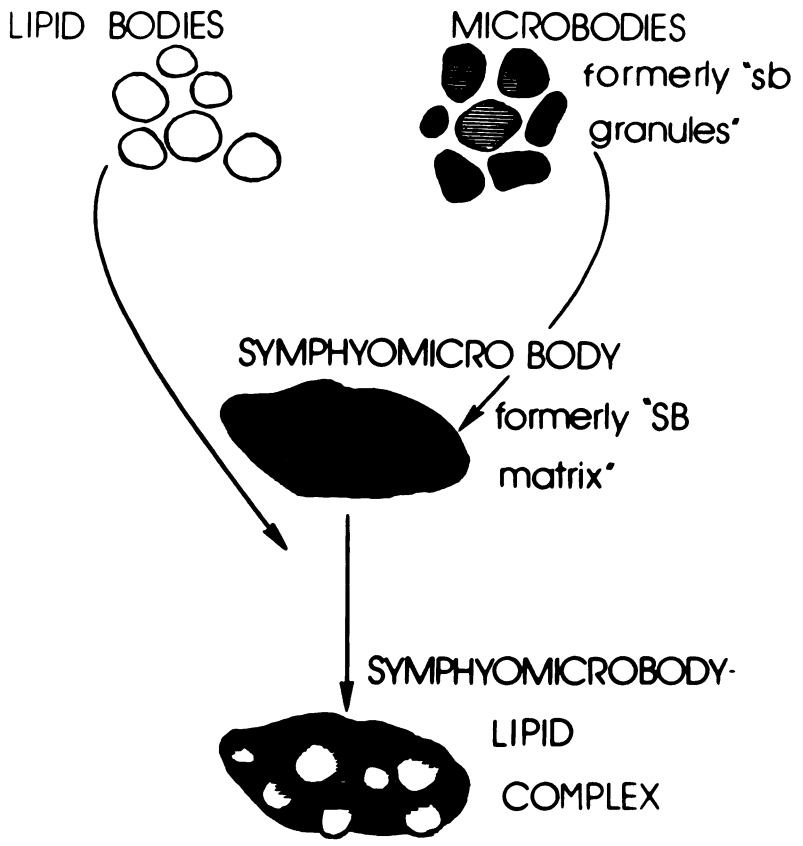


Fig. 12. The genesis of the symphyomicrobody--lipid complex in Blastocladiella emersonii. See text for details.

which it is constructed, i.e., the catalase positive sb granules of Lessie and Lovett (1968), are therefore microbodies.

What is the role and fate of the symphyomicrobody? The symphyomicrobody is closely associated with lipid particles and the mitochondrion; perhaps, therefore, it functions as a glyoxysome. Glyoxysomes play a central role in gluconeogenesis by virtue of their capacity to burn up fat and mediate the glyoxylate cycle *via* isocitrate lyase and malate synthetase, thus bringing about net synthesis of succinate which presumably finds its way to mitochondria where it is further metabolized (Beevers, 1969; Tolbert, 1971; Richardson, 1974). A key point in this operation is the fact that continuous activity of glyoxysomes requires the cooperation of outside enzymes (Müller et al., 1968; Beevers, 1969); therefore a metabolic interplay *via* movement of C_4 and C_6 molecules back and forth is thought to occur between glyoxysomes and mitochondria.

Isocitrate lyase activity was detected long ago (McCurdy and Cantino, 1960) in *B. emersonii* zoospore extracts; our work in progress has verified its presence, as well as that of malate synthetase, in spore populations. Preliminary data suggest that isocitrate lyase activity is associated with a particulate fraction of buoyant density 1.28 g/cm^3 on a sucrose gradient. However, there is not yet enough information available about other enzyme activities and metabolic properties of the symphyomicrobody of the zoospores of *B. emersonii* to establish if this giant organelle should be considered a glyoxysome or peroxisome or both---or, neither one.

If the symphyomicrobody behaves like a conventional glyoxysome, it presumably functions while a zoospore is swimming rather than during its encystment. Zoospores lose 64% of their triglycerides after 5 h of swimming, but there is no significant decrease in lipid when they encyst (Mills et al., 1974). Also, during swimming the symphyomicrobody decreases in size (Suberkropp and Cantino, 1973) whereas upon encystment it becomes somewhat branched (established from serial sections of zoospore populations induced to encyst synchronously; Cantino et al., unpublished data).

Little is known directly about the fate of the symphyomicrobody after germination. However, if isocitrate lyase were associated exclusively with the symphyomicrobody in the B. emersonii zoospore and with microbodies at other stages of its life history, then the available analytical data (McCurdy and Cantino, 1960) for total isocitrate lyase per plant could mean that new generations of microbodies are not produced until some 3-6 h after a zoospore has germinated. However, electron microscopic evidence to support or refute this possibility is not yet available.

The occurrence of microbodies and side-body complexes in other aquatic fungi. The three-dimensional shape of the symphyomicrobody in B. emersonii was established (Cantino and Truesdell, 1970) from studies of serial sections. Such serial section evidence has not been published, to our knowledge, for the shape of microbodies in other aquatic fungi (however, in this connection see Olson, 1973, below)---nor, for that matter, in other organisms. Furthermore, the size of the B. emersonii symphyomicrobody, as already emphasized (Suberkropp and Cantino, 1973), depends upon how long a zoospore has been swimming; similarly, this has not been established for microbodies in other aquatic fungi.

Within the past few years structural aggregates resembling either the whole side body complex in B. emersonii (i.e. a symphyomicrobody [SB]-lipid-mitochondrial complex bound by a backing membrane) or just the SB-lipid combination, have been seen in the zoospore of Blastocladiella britannica (Cantino and Truesdell, 1971) and other Chytridiomycetes. Especially noteworthy among the latter is the side body complex in the zoospores of Coelomomyces punctatus (Martin, 1971) which greatly resembles that in Blastocladiella emersonii. A somewhat comparable arrangement apparently occurs in two other species, Coelomomyces psorophorae (Whisler et al., 1972) and C. indicus (Madelin and Beckett, 1972). The latter authors also report a sequence of events in the formation of the side body complex in the sporangia of Coelomomyces similar to that which occurs during sporogenesis in Blastocladiella emersonii. A side body complex is also said to occur in the zoospores of Catenaria anguillulae (Chong and Barr, 1974). Similarly, an arrangement (made up of profiles termed 'Stüben bodies') resembling a side body complex has been observed in zoospores of Allomyces (Fuller and Olson, 1971); 'Stüben bodies' also reportedly occur in Phlyctochytrium arcticum (Chong and Barr, 1973). Recently, Olson (1973) concluded that the side body complex in the meiospores of Allomyces has a three dimensional organization resembling the one in Blastocladiella emersonii. Zoospores of Rozella allomycis also contain aggregates of "several lipid globules, a folded backing membrane, and a microbody" (Held, 1973), and microbody lipid complexes are said to occur in species of Entophlyctis and Rhizophydium (Chong and Barr, 1974). Finally, Harpochytrium hedinii zoospores contain

(Travland and Whisler, 1971) a tight association of lipid globules, a single rumposome, and profiles of electron dense granular material resembling sections through the SB-matrix in Blastocladiella emersonii.

Although the various profiles seen thus far in thin sections through fungal zoospores, as outlined above, resembles microbodies on structural grounds, supplementary evidence of a functional nature is now highly desirable. Furthermore, it will be most interesting to find out how many of them are formed by the process of symphyogenesis, and whether or not any of them decrease in size while zoospores are swimming as does the symphyomicrobody of B. emersonii, for these are characteristics that we have not encountered in the literature on microbodies. From these latter two points of view in particular, the B. emersonii symphyomicrobody apparently encompasses something more than that circumscribed by today's stereotype of the glyoxysome. However, if the symphyomicrobody is a glyoxysome, then the manner in which it is placed in the side body complex - in intimate contact with both the single mitochondrion, on the one hand, and the spore's total complement of lipid globules, on the other---illustrates what appears to be an exceptionally favorable arrangement for carrying out the supposed mission of a glyoxysome.

V

Isolation and Characterization of Microbodies and Symphyomicrobodies with Different Buoyant Densities from the Fungus *Blastocladiella emersonii*

Since the discovery of the functional nature of mammalian and leaf peroxisomes (DeDuve, 1966; Tolbert and Yamazaki, 1969) and glyoxysomes (Breidenbach and Beevers, 1967), there have been many publications on microbodies; they are summarized in a symposium (Hogg, 1969), a book (Hruban and Rechcigl, 1969), and reviews (Tolbert, 1971; Vigil, 1973; Richardson, 1974; Frederick et al., 1975). Plant microbodies, extensively characterized morphologically and biochemically (Vigil, 1973; Frederick et al., 1975), have been classified as glyoxysomes, peroxisomes and "unspecialized microbodies" (Huang and Beevers, 1971; Huang and Beevers, 1973; Huang, 1975). In contrast, descriptions of fungal microbodies have been far more limited (Frederick et al., 1975), identifications being based mainly on fine structural criteria. Analyses of isolated microbodies involving more than one enzyme have been limited to studies with *Saccharomyces* (Szabo and Avers, 1969), *Neurospora* (Kobr et al., 1969) and *Coprinus* (O'Sullivan and Casselton, 1973).

The zoospores and zoosporangia of the water mold *Blastocladiella emersonii* (Cantino and Hyatt, 1953) contain two kinds of catalase positive organelles, symphyomicrobodies and microbodies (Mills and Cantino, 1975). They are ontogenetically related in that several microbodies

give rise to a single large microbody by symphyogenesis. The purpose of this report is to provide statistically circumscribed data about the enzymatic activities of isolated symphyomicrobodies, and biochemical and physical supporting evidence for the method of origin of this unusual organelle.

MATERIALS AND METHODS

Synchronous cultures of B. emersonii were grown essentially as described (Lovett, 1967) except that synchronous sporogenesis was induced after 18 h at 22°C. Zoosporangia were collected by filtration at the time of papilla formation; spores were collected after filtration by centrifugation. Zoosporangia and spore pellets were suspended in a homogenizing medium consisting of 50 mM Na cacodylate, pH 7.5, containing 0.5 M sucrose, 2.5% Ficoll, 10 mM $MgCl_2$, 10 mM KCl, and 5 mM EDTA. Sporangia were broken by sonication (X3, 30 sec, 60 W, 4°C), with one min intervals between each treatment; spores were disrupted by passing through a 27 gauge hypodermic needle until they were broken as observed microscopically. Sporangial homogenates were passed through four layers of 8-ply cellulose gauze, and both the spore and sporangial homogenates were centrifuged at 300 x g for 7 min. The sporanigal supernatant was centrifuged at 10,000 x g for 15 min. The pellet was resuspended in homogenizing medium and applied to a continuous sucrose gradient, as was the 300 x g spore supernatant. The sucrose gradient (30-65%, w/w) was formed over a cushion of 65% (w/w) sucrose. All sucrose solutions contained 5 mM EDTA. After centrifugation for 4 h at 24,000 rpm (61,000 x g to 110,000 x g) in a swinging bucket rotor #969, model BD2 International ultracentrifuge,

fractions were collected by drops. All steps were done at 0-4°C. Sucrose concentrations were measured refractometrically; protein was determined colorimetrically (Lowry et al., 1951). Enzyme activities were followed spectrophotometrically at a ca 23°C using a Gilford model 222 photometer and model 2453 linear potentiometer recorder (Honeywell). The enzymes assayed and methods employed were: succinic dehydrogenase, EC 1.3.99.1 (Hiatt, 1961), fumarase, EC 4.2.1.2 (Racker, 1950), catalase, EC 1.11.1.6 (Beers and Sizer, 1952), isocitrate lyase, EC 4.1.3.1 (Dixon and Kornberg, 1959), and malate synthase, EC 4.1.3.2. (Ornston and Ornston, 1969). Specific activities are designated as μmoles of substrate used or product formed x min^{-1} x mg $protein^{-1}$.

RESULTS

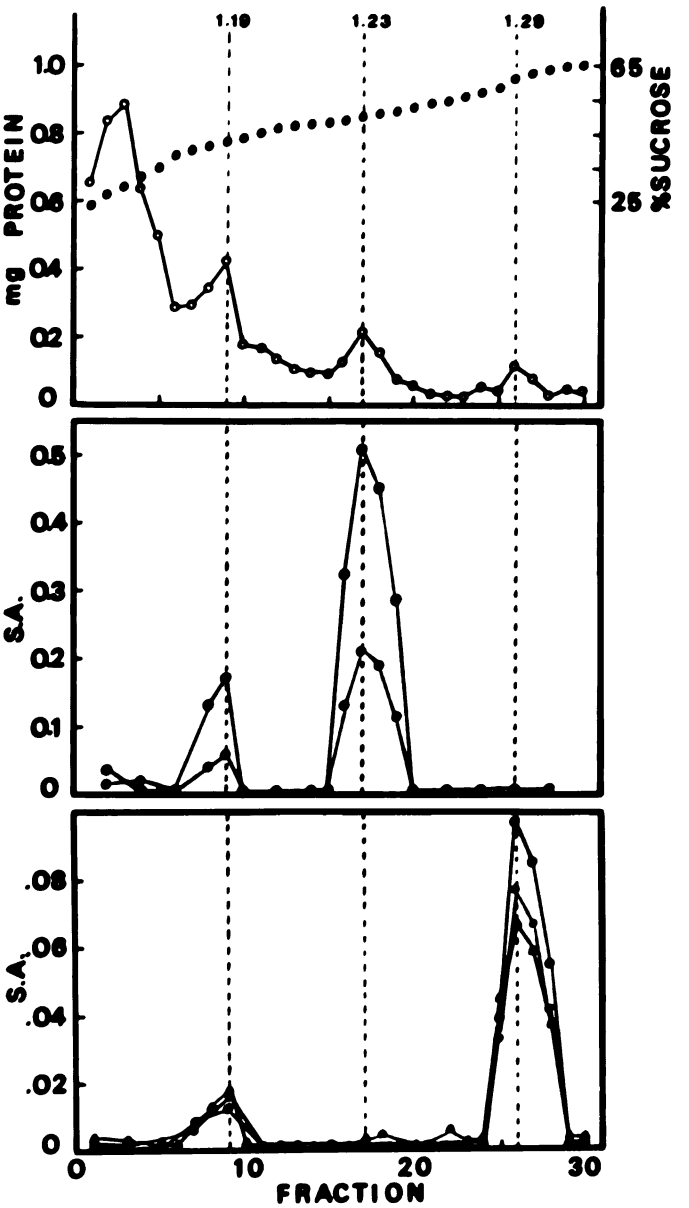
Of many techniques used to try to rupture spores with minimal organelle damage, passage through a syringe needle yielded the best results, with reproducible specific activities (Table 11). Most of the microbody and mitochondrial enzyme activities (ca 65% and 80%, respectively) were associated with particulates. Homogenates freed of large debris (300 x g) and centrifuged through sucrose density gradients yielded four protein peaks (Fig. 13, top). Fractions 1-5 contained soluble protein. Fractions 7-10 contained mitochondria-lipid-symphyomicrobody complexes (Mills and Cantino, 1975; Cantino and Mills, 1976) as determined both enzymatically and electron microscopically; they had a buoyant density of 1.19 g/cm^3. Fractions 16-19 (1.23 g/cm^3) were enriched for mitochondria (Fig. 14). The densest (1.29 g/cm^3) fractions, 25-28, contained symphyomicrobody material.

TABLE 11. Specific activities of enzymes associated with organelles, including their initial activities in the zoospore homogenates

	Symphyomicrobody enzymes			Mitochondrial enzymes	
	Isocitrate Lyase	Malate Synthase	Catalase	Succinic Dehydrogenase	Fumarase
S.A., spore homogenate	.0062 ± (*) .0028 (35)	.0091 ± .0057 (18)	1.368 ± .208 (10)	.0741 ± .0396 (18)	.0982 ± .0394 (12)
S.A., isolated organelle	.0642 ± .0358 (15)	.0913 ± .0304 (6)	19.520 ± 4.824 (6)	.1939 ± .0707 (15)	.4015 ± .1189 (6)
Maximum fold purification	25.0	16.2	19.2	5.2	6.0
Average fold	10.4	10.0	14.3	2.6	4.1

(*) Mean ± S.D.; number of separate experiments, in brackets.

Fig. 13. Distribution of organelle protein (top: o----o), and activities of mitochondrial enzymes (middle: fumarase, ●----●; succinic dehydrogenase, o----o), and microbody enzymes (bottom: catalase, ●----●, x 200; isocitrate lyase, o----o; and malate synthase, ▲----▲) after sucrose density gradient separation of components of the 300 x g supernatant from zoospores. Dashed lines mark the peaks for mitochondrion-lipid-symphyomicrobody complexes (1.19 g/cm^3), mitochondria (1.23 g/cm^3) and the symphyomicrobodies (1.29 g/cm^3).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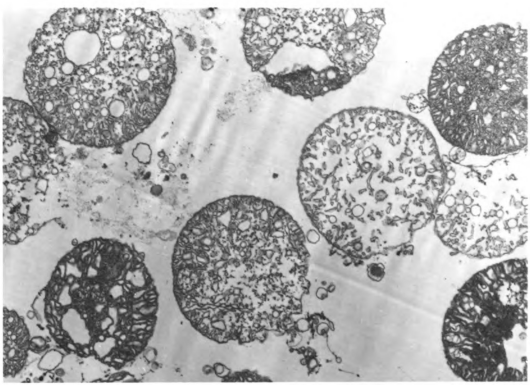

Fig. 14. Electron micrograph of isolated mitochondria, fixed in 0.5% glutaraldehyde and 0.5% OsO_4, and postfixed in 1% OsO_4, each for 1 h at 4°C, dehydrated, and embedded in Spurr's medium.

Isocitrate lyase, malate synthase, and catalase were associated with the symphyomicrobody fractions (Fig. 13, bottom), succinic dehydrogenase and fumarase with the mitochondrial fraction (Fig. 13, center). Table 2 shows mean buoyant densities of the symphyomicrobodies, microbodies, and mitochondria. Also listed (Table 11) are specific activities of enzyme markers associated with these isolated organelles, and the maximum and average purifications achieved for them.

The procedure used to isolate microbodies from sporangia yielded recoveries of ca. 60% for microbody enzymes in the 10,000 x g pellets, but only ca. 40% for mitochondrial enzymes. The profiles (Fig. 15) show that a shift in density (Table 12) occurred as the symphyomicrobody was fused out of smaller microbodies during zoosporogenesis. The profile (Fig. 15, top) for isocitrate lyase from sporangia collected after cleavage into spores but before spore release contrasts sharply with the profile (Fig. 15, bottom) for isocitrate lyase from sporangia harvested at the time of papilla formation, i.e., just before sporogenesis when symphyomicrobodies had not yet formed. The distribution of catalase and malate synthase followed that of isocitrate lyase; the shift in density measured with these three markers was also demonstrable in discontinuous sucrose gradients (not shown). The mitochondrial enzyme markers derived from sporangia undergoing papilla formation were located at a buoyant density of ca. 1.20 g/cm^3.

DISCUSSION

The zoospores of B. emersonii contain a single large symphyomicrobody (Mills and Cantino, 1975; Cantino and Mills, 1976), so called because it is formed by the union of small microbodies (formerly "sb granules";

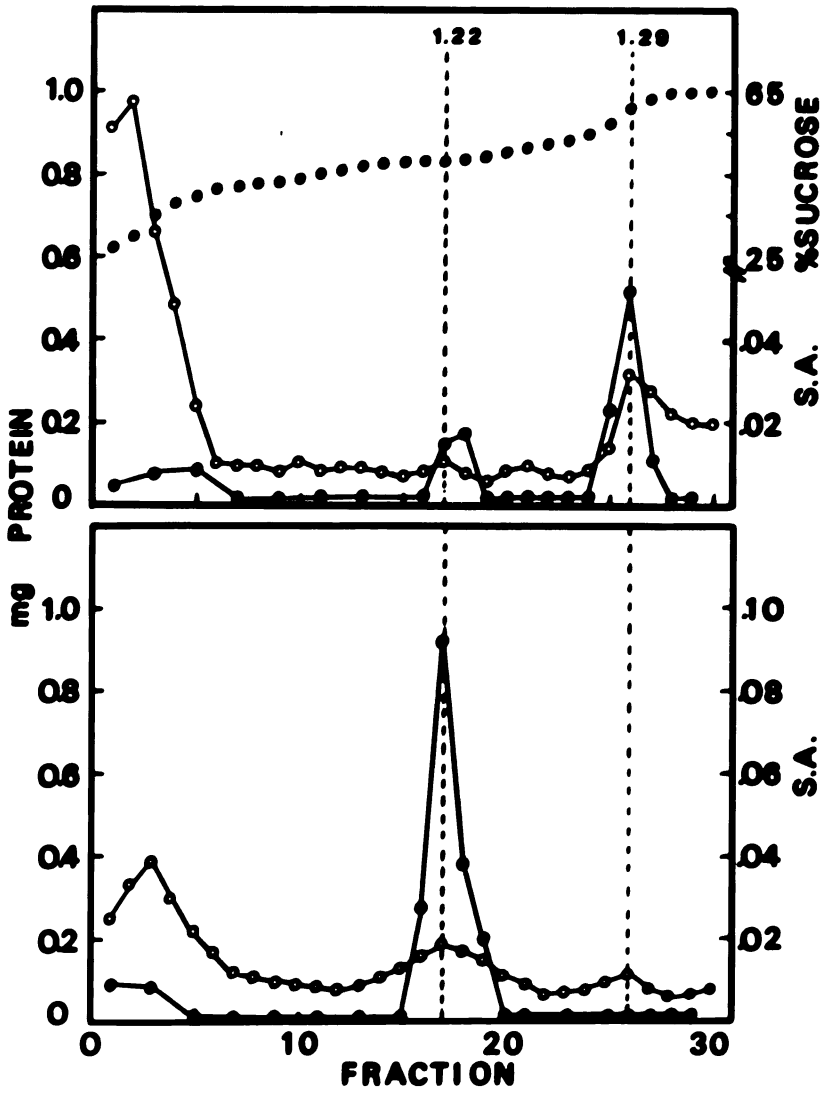

Fig. 15. Distribution of protein (o----o) and isocitrate lyase activity (●----●) after sucrose density gradient separation of components of the 10,000 x g pellet from zoosporangia. The dashed lines show the shift in density of the peak activities for symphyomicrobodies (top) and individual microbodies (bottom).

TABLE 12. The buoyant densities of isolated organelles

Symphyomicrobodies	Microbodies	Mitochondria
1.292 ± .0121 (*)	1.222 ± .006	1.219 ± .010
(15)	(5)	(15)

(*) g/cm^3; mean ± S.D.; number of experiments, in brackets.

Lessie and Lovett, 1968) during sporogenesis (Mills and Cantino, 1975; Cantino and Mills, 1976). The small microbodies isolated from zoosporangia just before sporogenesis have a mean buoyant density of 1.222 g/cm^3, are 0.2 - 0.3 μ in diam, and carry catalase, isocitrate lyase, and malate synthase activities. However, if microbodies are isolated from zoosporangia 2 h later, at the time of sporogenesis but before spore release, two changes associated with symphyogenesis occur: a large increase in microbody size, and an increase in buoyant density to 1.292 g/cm^3.

This increase in density may result from elevated sucrose uptake due to the larger size of the new organelle, or from addition of proteins at the time of symphyogenesis. Changes in buoyant density have been reported for microbodies in other organisms. The density of developing wheat leaf peroxisomes increases from 1.18 g/cm^3 to 1.25 g/cm^3 (Feierabend and Beevers, 1972), the explanation offered being acquisition of new protein. Shifts in microbody density in Arum (Berger and Gerhardt, 1971) and developing soybean cultures (Moore and Beevers, 1974) have also been described. Changes in microbody size have also been detected, e.g., in Phaseolus (Gruber et al., 1973). However, in none of these or other instances (Vigil, 1973; Frederick, 1975) has a fusion of microbodies been involved.

The B. emersonii symphyomicrobody has been shown ultracytochemically (Mills and Cantino, 1975) and biochemically (this report) to contain catalase, hence it might be classified as a peroxisome. But since it also carries malate synthase and isocitrate lyase, it might be functioning as a glyoxysome. This idea is supported by indirect evidence, such as

the close physical relationship (Cantino and Mills, 1976) among lipid globules, mitochondrion and symphyomicrobody. Furthermore, 64% of the triglycerides in zoospores are used after 5 h of swimming (Mills et al., 1974); they are thought to be the major component of the lipid globules, which also decrease (Suberkropp and Cantino, 1973). When we analyzed homogenates of spores that had been swimming 5 h, the two glyoxylate cycle enzyme activities had increased three-fold whereas catalase had not changed.

If the B. emersonii symphyomicrobody is a glyoxysome, how does it compare with microbodies isolated from other fungi? There is a paucity of information about isolated fungal microbodies. What little there is indicates that at least some glyoxylate cycle enzymes are associated with particulates. The latter vary considerably, however, in buoyant density and enzyme content. In yeast (Szabo and Avers, 1969) and Coprinus (O'Sullivan and Casselton, 1973), particulates containing glyoxylate cycle enzymes had lower buoyant densities than mitochondrial fractions. Isocitrate lyase from yeast was bimodally distributed between mitochondrial and microbody fractions but malate synthase was almost entirely associated with the microbody fraction (Szabo and Avers, 1969). In Neurospora (Kobr et al., 1969) both malate synthase and isocitrate lyase were associated with particulates of greater buoyant density than that carrying mitochondrial enzymes. The particulates from Coprinus and Neurospora were classified as glyoxysomes; the yeast organelle was called a peroxisome since catalase and glycolate oxidase were associated with it. We think the symphyomicrobody of B. emersonii zoospores may be functioning as a glyoxysome.

Although plant microbodies seem to develop from the endoplasmic reticulum (Vigil, 1973; Frederick et al., 1975) it is also a fact (Truesdell and Cantino, 1971) that at one stage in the life cycle of B. emersonii, after a zoospore encysts, microbody-like structures are generated by fission of the symphyomicrobody. Further studies of the formation and decay of this novel organelle should contribute new insights on microbody ontogeny. Work is in progress to further characterize the symphyomicrobody chemically, enzymatically and physiologically.

DISCUSSION

The identification of a structure, based entirely on morphology and without knowledge of its function or origin, often leads to confusion in terminology. Such is the case for the unit membrane bound organelle labeled "sb granule" in the sporangia and zoospores of B. emersonii (Lessie and Lovett, 1968), "SB matrix" in zoospores of B. emersonii (Cantino and Truesdell, 1970), and "Stüben body" in zoospores of Allomyces (Fuller and Olson, 1971). The function of this organelle was not known from any direct evidence. The present study has shown that this structure closely resembles a class of organelles broadly termed microbodies.

Earlier enzymological and physiological data suggested that this organelle might be functioning in two possible ways, either as a lysosome or as a storage organelle. Cantino and Mack (1969), while studying gamma particles in B. emersonii zoospores, reported that the lipid particles in the side body complex were acid phosphatase positive. Acid phosphatase is characteristically associated with lysosomes (DeDuve and Wattiaux, 1966). Suberkropp and Cantino (1973) observed a loss in both lipid and polysaccharide as zoospores of B. emersonii swam in non-nutrient media. They also noted ultrastructural differences when zoospores that had been swimming for 10 hours were compared to zero time spores. After 10 hours of swimming the number and size of the lipid bodies had decreased and the SB matrix was thinner.

These observations led to two routes, one negative, the other positive. When zoospores were tested for acid phosphatase activity, using a sensitive cytochemical test (Butterworth, 1971), the only reaction

product detected was in the anterior portion of the zoospore, not the posterior region where the side body complex is located (Fig. 16, Appendix A). This seemed to rule out the possibility of the SB matrix being a lysosome. However, preliminary data on lipid utilization as zoospores swam supported the conclusion that the SB matrix was acting directly as a lipid reservoir or else was involved indirectly in lipid utilization.

The metabolism of lipids seemed to be a key requirement for understanding the function of the SB matrix, so my first step was to characterize the lipids in zoospores of B. emersonii. The zoospores contain between 10-13% total lipid (Suberkropp and Cantino, 1973; Smith and Silverman, 1973; Mills and Cantino, 1974). All the major classes of lipids (neutral lipids, glycolipids and phospholipids) were found (Table 1; Smith and Silverman, 1973). Differences in composition of these classes were observed when comparing spores derived from plates with those from liquid culture. Plate-grown spores contained 46.6% neutral lipid, 15.8% glycolipid and 37.6% phospholipid, while spores produced in liquid culture contained 28.3% neutral lipid, 12.0% glycolipid and 59.7% phospholipid. The results I obtained for liquid-culture spores are in good agreement with data presented by Smith and Silverman (1973) for liquid-culture spores of B. emersonii. They reported that the spores contained 30.0% neutral lipid, 15.0% glycolipid and 55.0% phospholipid.

Qualitatively, the individual lipid components for both plate culture and liquid culture spores were the same. PC and PE were the major phospholipids, TG the major neutral lipid, and DGDG the major glycolipids. Quantitatively, however, differences in composition of the individual lipid

components were observed when the two culture techniques were compared. PC, TG and DGDG were approximately the same for both; 20.3% vs. 23.9%, 13.5% vs. 18.5% and 13.3% vs. 10.2%. The main difference was in PE. It accounted for only 6.7% of total lipid from plate-derived spores as opposed to 27.8% of total lipid from liquid-culture-derived spores. It is thought that these differences in lipid composition are due to exogenous oxygen deficiencies and corresponding shifts to fermentative metabolism (see discussion at the end of Experimental, I).

Once the lipid composition of zoospores was established, I followed lipid changes that occurred during development. When zoospores were allowed to swim in a non-nutrient medium for 5 hours, total lipid decreased at a rate of 0.68 pg/5 hours as compared to 0.94 pg/5 hours as reported by Suberkropp and Cantino (1973). The greatest change for any one lipid component for this period was the loss in TG. The TG decreased 64% after 5 hours; it was probably being used as an energy source. When zoospores were further incubated for an additional five hour period the TG increased slightly, while PE-LPE and PC-LPC decreased by 61 and 39%, respectively. These phospholipids were probably being used directly for energy and indirectly for TG biosynthesis.

The incubation of zoospores of B. emersonii in a non-nutrient medium closely resembles the starvation conditions imposed on Tetrahymena pyriformis (Levy and Elliott, 1968). Both organisms utilize TG. In Tetrahymena the TG was probably used for energy (Levy and Elliott, 1968) or for synthesis of phospholipid (Borowitz and Blum, 1976). The involvement of phospholipid directly for energy and indirectly for TG biosynthesis has been reported for Tetrahymena pyriformis (Levy and Elliott, 1968) and developing soybean cotyledons (Wilson and Rinne, 1976).

When zoospores were induced to encyst and analyzed for lipid, I found that total lipid did not change significantly. This is in sharp contrast to results obtained by Smith and Silverman (1973). They reported a 67% loss in lipid when zoospores germinated. However, they stated that the spores were still motile and still contained well defined nuclear caps, which indicated that they were not analyzing only encysted spores.

Upon encystment all glycolipid components decreased. One of the PGDG was completely utilized, while MGDG, DGDG and the other PGDG decreased by half. After five hours of growth the glycolipids had returned to their original level. Striking increases in phospholipid, MG and SE were noted for the 5 h germlings. The increase in phospholipid was probably due to membrane synthesis, while MG was most likely used for synthesis of more complex lipid components (Jack, 1965). The reason for the increase in SE is not known although it may have paralled nuclear division. Sterol esters are thought to be major lipid constituents of nuclear membranes (Sato et al., 1972). Similar increases in SE have been reported to occur during sorocarp formation by Dictyostelium discoideum (Long and Coe, 1974), ascospore development in Saccharomyces cerevisiae (Illingworth et al., 1973) and spherulation in Physarum polycephalum (Kleinig et al., 1975). In Physarum polycephalum sterol accumulation is paralleled by triglyceride accumulation (Kleinig et al., 1975) and it is thought that these lipids are located in droplets which are enriched during spherulation (Zaar and Kleinig, 1975).

When the lipid globules in the zoospore of B. emersonii were isolated, I found that they were composed mainly of TG and FS, accounting for 84.5%

and 7.4% of the total dry weight of the lipid globules. Smaller amounts of DG, γ-carotene, FFA, phospholipid and protein were found. No SE, MG or glycolipids were detected.

The TG associated with lipid globules represents 89% of the total zoospore TG. This TG was probably used as a source of energy, since 64% of the TG decreased after zoospores swam for 5 hours under starvation conditions. These data support the ultrastructural observations reported by Suberkropp and Cantino (1973) who, as stated previously, found a decrease in number and size of lipid globules as the zoospores swam.

A parallel situation was observed for liver cells of starved rats. Ashworth et al. (1966) showed ultrastructurally that there was a marked decrease in the number of lipid droplets in hepatocytes of starved rats. DiAugustine et al. (1973) later observed a decrease in TG when these lipid droplets were isolated.

The loss of lipid as zoospores swim and the close resemblance of the SB matrix to microbodies (see Introduction) indicates that the SB matrix might be functioning as a glyoxysome. Glyoxysomes are a special type of microbody (DeDuve, 1969) that function in the conversion of lipid to carbohydrate (Tolbert, 1971; Richardson, 1974). Microbodies have been identified by using ultracytochemical techniques (see Vigil, 1973; Frederick et al., 1975). Catalase seems to be a characteristic enzyme for microbodies (DeDuve, 1969) and can be demonstrated ultracytochemically (Novikoff and Goldfischer, 1968) using 3,3'-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 as a substrate.

When zoospores of B. emersonii were incubated in the DAB/H_2O_2 reaction mixture, the osmium black reaction product was localized in

the SB matrix. The reaction product was not detected in any other organelle. Cytochemical localization of catalase in microbodies has been reported for: Saccharomyces cerevisiae (Hoffmann et al., 1970), Hansenula polymorpha (Dijken et al., 1975), Candida tropicalis (Osumi et al., 1975), Phytophthora palmivora (Philippi et al., 1975) and various n-alkane grown and methanol-utilizing yeasts (Osumi et al., 1974; Fukui et al., 1975). Microbodies have also been recently identified ultracytochemically in another water mold, Entophlyctis (Powell, 1976).

The sporangia of B. emersonii contain sb granules which are associated with lipid particles (Lessie and Lovett, 1968). The sb granules become aligned along side the mitochondrion at time of zoosporogenesis to form a continuous structure, the SB matrix, in the zoospores (Cantino and Truesdell, 1970). When sporangia were tested ultracytochemically the sb granules were catalase positive. The sb granules are, therefore, microbodies which come together during zoosporogenesis to form the symphyomicrobody (formerly the SB matrix) by means of symphyogenesis (see Fig. 12 for details).

It was demonstrated that microbodies and symphyomicrobodies isolated from sporangia and zoospores of B. emersonii contain catalase, isocitrate lyase and malate synthase. The microbodies had an equilibrium density of 1.222 g/cm^3 while symphyomicrobodies had an equilibrium density of 1.292 g/cm^3. Both the microbodies and symphyomicrobodies were clearly separated from one another and had a greater equilibrium density than the mitochondrial enzyme markers, succinic dehydrogenase and fumarase.

Microbodies have been isolated from other fungi and there seems to be much variation in the types of enzymes found associated with these

organelles, their buoyant densities, and whether they are classified as microbodies or peroxisomes. Szabo and Avers (1969) were the first to report isolation of microbodies from fungi. They isolated unit membrane bound particles from the fungus Saccharomyces cerevisiae that contained the enzymes catalase, glycolate oxidase, malate synthase and isocitrate lyase. These particles had a lower buoyant density than mitochondria and were labeled peroxisomes (Szabo and Avers, 1969; Avers, 1971), since they contained catalase and at least one H_2O_2 producing oxidase . They suggested, however, that the microbody was functioning as a glyoxysome, since glyoxylate cycle enzymes were present. Perlman and Mahler (1970), on the other hand, found isocitrate lyase to be totally nonparticulate in Saccharomyces cerevisiae, a conclusion recently supported by Parish (1975). Parish (1975) also was unable to detect malate synthase activity in the peroxisomal fraction from Saccharomyces cerevisiae, but did find in addition to catalase the other peroxisomal enzymes urate oxidase, D-amino acid oxidase, and L-α-hydroxy acid oxidase (glycollate oxidase). He also reported that the peroxisomes from this organism were more dense than the mitochondria, another difference between his results and those of Szabo and Avers (1969).

Microbodies containing crystalloids have also been isolated from the yeast-like phase of a Kloeckera sp. (Fukui et al., 1975). These microbodies had a greater buoyant density than mitochondria and contained a flavin-dependent alcohol oxidase, as well as the characteristic microbody enzymes, catalase and D-amino oxidase. U-bodies in Phytophthora palmivora (Philippi et al., 1975) were identified as microbodies, based on the cytochemical demonstration of catalase in these organelles and the association of catalase with a particulate fraction.

Microbodies, identified as either peroxisomes or glyoxysomes, have been isolated from the cellular slime mold Dictyostelium discoideum (Parish, 1975) a species of the water mold Entophlyctis (Powell, 1976), the ascomycete Neurospora crassa (Kobr et al., 1969) and the basidiomycete Coprinus lagopus (O'Sullivan and Casselton, 1973). The peroxisomes of Dictyostelium discoideum (Parish, 1975) were less dense (1.19 g/cm^3) than mitochondria (1.21 g/cm^3) and contained catalase and urate oxidase. Glyoxysomes were found in Entophlyctis sp. and Coprinus lagopus (Powell, 1976; O'Sullivan and Casselton, 1973). The glyoxylate cycle enzymes malate synthase and isocitrate lyase were associated with these microbodies. The glyoxysomes of Coprinus were less dense than mitochondria, while the glyoxysomes of Entophlyctis were more dense. Glyoxysome-like particles have also been isolated from Neurospora crassa (Kobr et al., 1969). These microbodies contained isocitrate lyase and malate synthase and had a buoyant density of 1.21 g/cm^3 as compared to mitochondria which had a buoyant density of 1.18 g/cm^3.

When these microbodies, and microbodies isolated from other organisms, are compared to microbodies and symphyomicrobodies of B. emersonii, a number of similarities and differences are notable. The microbodies within the sporangia and symphyomicrobodies in zoospores of B. emersonii are unit membrane bound and have a finely granular matrix, characteristics shared by microbodies in general (Hruban and Rechcigl, 1969). However, the microbodies in the sporangia are smaller than most microbodies (0.2 μm - 0.3 μm in diameter, as compared to 0.5 μm - 1.5 μm), while the symphyomicrobody is some 3-4 times larger than most microbodies. Microbodies and symphyomicrobodies from B. emersonii contain catalase, isocitrate

lyase and malate synthase (these enzymes are generally associated with glyoxysomes; Breidenbach and Beevers, 1967) and are found next to lipid globules, also a characteristic of glyoxysomes (Vigil, 1973).

An important difference between symphyomicrobodies and microbodies is the method of formation of these two organelles. Symphyomicrobodies are formed by symphyogenesis, i.e. the union of formerly separate microbodies to form one larger microbody. Microbodies are generally thought to be formed either directly from endoplasmic reticulum (Vigil, 1973; Richardson, 1974; Frederick et al., 1975) or from pre-existing microbodies (White and Brody, 1974; Osumi et al., 1975). Most of the chemical data support the hypothesis that microbodies are formed by endoplasmic reticulum.

Kagawa et al. (1973) using [^{14}C]-choline showed that newly synthesized lecithin was first found in endoplasmic reticulum and then incorporated into glyoxysomal membranes. Both Donaldson (1976) and Lord (1976), independently, found endoplasmic reticulum to be the major site of membrane proliferation in castor bean endosperm. Donaldson (1976) also reported the appearance of labeled phospholipid and diglycerides in glyoxysomes subsequent to their appearance in endoplasmic reticulum. Bowden and Lord (1976a) found a similarity in polypeptide composition between endoplasmic reticulum and glyoxysomal membranes. They also observed that labeled [^{35}S]-methionine was first incorporated into endoplasmic reticulum, then later into glyoxysomes (Bowden and Lord, 1976b). Gonzalez and Beevers (1976) found a strong antigenic response in the endoplasmic reticulum when it was challenged by antiglyoxysomal-protein antiserum. These data all support the view that microbodies are derived from endoplasmic reticulum.

The zoospores of B. emersonii do not contain endoplasmic reticulum (Cantino et al., 1963) but it is found in the sporangia (Lessie and Lovett, 1968). Microbodies in such sporangia could be formed from endoplasmic reticulum. A close association between a microbody and endoplasmic reticulum can be seen in Fig. 11. It is also known that the symphyomicrobody fragments when zoospores encyst, forming separate microbodies (Cantino, unpublished data). The formation of the symphyomicrobody, accompanied by an increase in size and buoyant density (see Experimental, V), and genesis of microbodies by fragmentation, are both unusual when considering present ideas about microbody biogenesis. Another exceptional feature about the symphyomicrobody is the decrease in its size as the zoospore swims (Suberkropp and Cantino, 1973), a characteristic not encountered in the literature on microbodies.

Further work is needed to explain these unusual features. Also, additional information is needed to establish the ontogenetic and functional relationship between the microbodies in the sporangia and symphyomicrobodies in the zoospores of B. emersonii. Chemical characterization, enzymology, and enzyme localization at different developmental stages could provide valuable information about the origins (see Appendix B for preliminary data), fates and interrelationships of these organelles.

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APPENDIX A

APPENDIX A

Cytochemical Localization of Acid Phosphatase in Zoospores of B. emersonii

Cantino and Mack (1969) reported that the lipid particles in zoospores of B. emersonii were acid phosphatase positive. The lipid particles are components of the side body complex (Cantino and Truesdell, 1970). Acid phosphatase has been observed in fungal spherosomes (Armentrout et al., 1968), spherosome-like bodies (Hislop et al., 1974), lysosomes (Wilson et al., 1970) and vacuoles (Hänssler et al., 1975; Armentrout et al., 1976).

The purpose of this study was to verify the site of acid phosphatase activity in the side body complex and to determine whether or not acid phosphatase could be used as an enzyme marker for the isolation of any one of these organelles.

Zoospores of B. emersonii were cultured at 22°C in the dark on Difco PYG agar in standard Petri plates. At the time of spore release plates were flooded with 5 ml of 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The spores were filtered 10 min. later and concentrated by centrifugation (1000 xg for 5 min). Zoospore pellets were fixed for 1 h in buffered 1% glutaraldehyde at 4°C. After fixation, the pellets were rinsed with 50 mM sodium acetate buffer, pH 4.5, and incubated 30 min at 37°C in a reaction mixture which contained 4 mM lead acetate, 3 mM p-nitrophenyl phosphate and 50 mM sodium acetate buffer, pH 4.5. The zoospore pellet was then rinsed in buffer and placed in 2% ammonium sulfate for 5 min, rinsed in buffer, and a small amount of the spore suspension was mounted on a microscope slide and viewed with a Ziess

Photomicroscope II. Acid phosphatase activity was indicated by dark brown to black deposits of lead sulfide. Controls were treated as above, but the substrate, p-nitrophenyl phosphate, was omitted.

Results of the cytochemical test are shown in Fig. 16. The lead sulfide deposits (as indicated by arrows, left top and bottom) were localized in the anterior region of the zoospore, not the posterior portion where the side body complex is located. No reaction product was found in the controls (top and bottom right). The acid phosphatase is probably cytoplasmic since the only organelles in this region of the zoospore are the γ-particles, which do not have acid phosphatase activity (Myers and Cantino, 1974).

There may be several possible explanations for the apparent difference in the results discussed above; for example, previous history of the spores, differences in staining procedure, etc. The question has not been resolved.

APPENDIX B

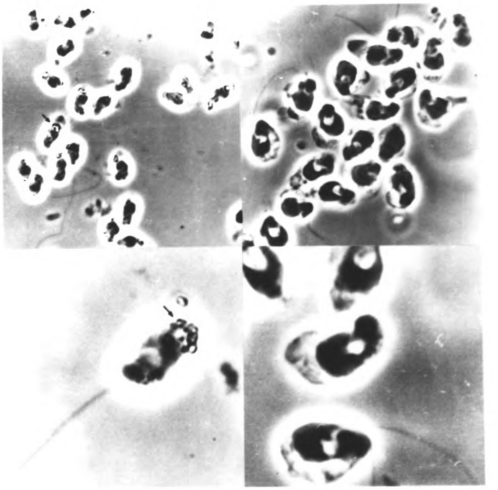

Fig. 16. The response to the Gomori lead nitrate cytochemical procedure for localization of acid phosphatase activity.

APPENDIX B

Mitochondrial and Microbody Enzyme Activity During Development of B. emersonii

McCurdy and Cantino (1960) while studying isocitrate lyase in B. emersonii reported that its activity per plant did not increase until some 3-6 hours after zoospore germination. Similar results were reported for some tricarboxylic acid cycle enzymes in B. emersonii (Khouw and McCurdy, 1969; Ingebretsen and Sanner, 1976). In this study, I tracked changes in the microbody enzyme isocitrate lyase and the mitochondrial enzymes succinic dehydrogenase and fumarase with the hope of identifying the developmental stage at which new populations of microbodies and mitochondria were produced.

Synchronous liquid cultures of B. emersonii were grown by standard procedures (Lovett, 1967) except that sporogenesis was induced at 18 h of growth at 22°C. Zoospores were collected (after filtration) by centrifugation, cysts and germlings by centrifugation, and sporangia by filtration. Cell homogenates were prepared by sonication (30 sec intervals, 60 W at 4°C) until broken as established microscopically. The homogenizing medium consisted of 50 mM sodium cacodylate, pH 7.5, containing 0.5 M sucrose, 2.5% Ficoll, 10 mM $MgCl_2$, 10 mM KCl and 5mM EDTA. Isocitrate lyase, succinic dehydrogenase and fumarase were measured spectrophotometrically at 23° (see Experimental, V for details). Specific activities are designated as μmoles of substrate used or product formed x min^{-1} x mg $protein^{-1}$.

Microbody and mitochondrial enzyme activities are given in Table 13. Upon encystment the specific activities of both isocitrate lyase and

TABLE 13. Mitochondrial and microbody enzyme activities during different developmental stages of B. emersonii

Developmental Stage	pg Protein/ Plant	Specific Activities ($x10^3$) Isocitrate lyase	Succinic dehydrogenase	Fumarase	Activity/plant ($x10^{15}$) Isocitrate lyase	Succinic dehydrogenase	Fumarase
Zoospore	21.46	3.3	29.8	59.4	.038	.342	.681
Cyst	23.64	1.3	23.8	10.3	.017	.325	.141
3 h germling	37.52	3.4	20.8	30.3	.058	.365	.530
6 h plant	71.11	1.3	16.6	28.1	.092	1.18	1.99
9 h plant	97.50	1.1	15.2	27.3	.108	1.68	2.67
12 h plant	265.77	1.3	16.8	19.2	.342	4.47	5.12
15 h plant	1421.60	1.9	17.7	25.3	2.78	25.18	36.03
18 h plant	2117.81	2.3	26.4	46.5	4.89	56.07	91.38
Cleaved plant	1842.41	3.2	33.3	49.6	5.95	61.32	96.34

fumarase decreased. This is also reflected in the activities per plant. However, the specific activity and activity per plant for succinic dehydrogenase changed very little. During the first nuclear divisions (3 h germlings) the specific activities of fumarase and isocitrate lyase increased, while the specific activity of succinic dehydrogenase decreased; then, the specific activities of all three enzymes remained roughly constant. At induction the specific activities of all three enzymes increased, and after zoosporogenesis they had again reached levels close to the specific activities characteristic of the zoospores.

Changes in enzyme activities per plant paralled changes in protein per plant after encystment. There was first a lag in protein synthesis and enzyme development, then a log phase between 9 and 18 hours of growth. At 18 hours the sporangia were induced to form papillae by removal of the spent nutrient medium. Between 18 and 24 hours the sporangia underwent cytodifferentiation and produced zoospores. Also during this time there was a decrease in total protein per plant and only slight increases in enzyme activity per plant.

These results are in good agreement with those reported for isocitrate lyase (McCurdy and Cantino, 1960), fumarase and succinic dehydrogenase (Khow and McCurdy, 1969) during development of B. emersonii. The specific activities I report here are also close to those reported earlier for the three enzymes (see McCurdy and Cantino, 1960; Khouw and McCurdy, 1969). The loss in protein after induction (Table 13) has also been observed by Lodi and Sonneborn (1974) and Lovett (1975).

Further work will have to be done to determine the significance of the lag in enzyme synthesis during the first 9 hours of growth, and the subsequent increase in microbody and mitochondrial enzymes. It could mean that new populations of microbodies and mitochondria are being formed at this time. If this is true, it will be an excellent time for studying microbody biogenesis.