THE RELATION BETWEEN THE PHYSICAL-CHEMICAL PROPERTIES OF THE ZOOSPORE PLASMA MEMBRANE AND DIFFERENTIATION IN *BLASTOCLADIELLA EMERSONII*

By

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ABSTRACT

THE RELATION BETWEEN THE PHYSICAL-CHEMICAL PROPERTIES OF THE ZOOSPORE PLASMA MEMBRANE AND DIFFERENTIATION IN *BLASTOCLADIELLA EMERSONII*

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Blastocladiella emersonii is an excellent model system for examining the role(s) of membranes in cellular differentiation. The zoospores are highly differentiated cells which can undergo rapid morphological changes in response to their environment. Cations and temperature play important role(s) in these changes, K^+ ions inducing and Ca^{++} ions inhibiting encystment, in a temperature dependent manner. These developmental changes do not seem to involve either protein or RNA synthesis, but do involve extensive membrane alterations. The first section of this dissertation establishes a correlation between the physiological parameters involved in encystment and the physical-chemical properties of the zoospore plasma membrane, *in vivo*. Intact zoospores and zoospore total lipid extracts were spin-labeled and examined with electron spin resonance spectroscopy. Three breaks points in plots of the hyperfine splitting parameter ($2T_{||}$),

order parameter (S), and partition parameter (f) vs. temperature were observed. The first and third breaks (T_L and T_H) were independent of K^+, Ca^{++}, or Mg^{++} ion concentrations, similar to the breaks observed in lipid extract dispersions, and correlated well with the temperature limits of zoospore viability. In contrast, the middle break point (T_M) was affected by Ca^{++} ions, K^+ ions reversed the Ca^{++} ion effect. The cation-induced changes in T_M were closely correlated with the temperature dependence and physiological effects of cations on encystment.

The second section expanded upon these results to determine which lipid components were responsible for the observed phenomenon. Lipid dispersions made from various combinations of zoospore glyco-, phospho-, and neutral lipid components indicated that the phase transformation (T_L to T_H) observed were due to the glycolipid, and not the phospholipid fraction. This transformation seems to represent a gel-to-liquid-crystalline phase transition.

Ca^{++} ions increased the $2T_{||}$ values of glycolipid, but not the phospholipid dispersions. The inclusion of neutral lipids did not affect this $2T_{||}$ increase in glycolipid dispersions, but was eliminated at a 5 to 1 phospholipid/glycolipid ratio. K^+ ions neither prevented

or reversed the Ca^{++} ion-induced $2T_{||}$ increase in glycolipid dispersions.

The third section described a procedure for the isolation of zoospore plasma membranes, by rupturing the plasma membrane. Analysis of the lipids present indicated that the plasma membrane was composed of diglucosyldiglycerides (47% of total lipid) and neutral lipids, rather than phospholipids. No triglycerides were detected. The preponderance of glycolipids in the plasma membrane united the first two sections above.

The fourth section details the properties of the middle break point, T_M, in isolated plasma membranes. T_M was determined to be the result of a lipid/lipid rather than lipid/protein interaction. Ca^{++} ions affected this interaction in the same manner as observed <u>in vivo</u>. K^+ ion addition had no affect on T_M, in the absence of ATP. However, after including ATP, K^+ ions did reverse the Ca^{++} ion effect. ATP was found to generate an "energized membrane" state which K^+ ions could depolarize. We suggest that the K^+ ion induced reversal of the Ca^{++} ion effect, and therefore the changes in the lipid/lipid interaction responsible for T_M, is a consequence of the K^+ ion induced depolarization of the membrane potential.

"When I examine myself and my methods of thought, I come to the conclusion that the gift of fantasy has meant more to me than my talent for absorbing positive knowledge."

A. Einstein

ACKNOWLEDGEMENTS

I wish to express my appreciation to the members of my committee, Dr. E. C. Cantino, who by his discovery and extensive studies of B. emersonii laid the foundation for my studies, and Dr. A. Haug, in whose laboratory I learned the theoretical and experimental principles of membrane biology. I am also indebted to Dr. K. Poff and Dr. D. Delmer for their advice (scientific and otherwise) during the course of my studies.

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GENERAL INTRODUCTION

The role(s) of the plasma membrane in development and differentiation is presently an area of intense interest in biology. Unfortunately, most of the existing body of knowledge on plasma membranes is derived either from studies on prokaryotes, erythrocytes, and model membranes, where the difficulties associated with eukaryotic systems are avoided, or from studies on Saccharomyces cerevisiae (Durán et al., 1975; Santos et al., 1978) and Neurospora crassa (Scarborough, 1975), where a cell wall must first be removed, either enzymatically or by use of cell surface mutants. A desirable alternative would be to find an organism which 1) exhibits the differentiation and development characteristic of an eukaryotic organism, and 2) is structurally organized so as to minimize possible contamination from cytoplasmic organelles, and potential artifacts related to cell wall removal.

Zoospores of the chytridiomycete Blastocladiella emersonii fulfill both of these criteria, and are an excellent model system for examining the role(s) of the plasma membrane in cellular differentiation. There are

three properties of the zoospore in particular which strongly recommend its use for membrane studies. They are: 1.) the ultrastructural organization of the zoospore, 2.) the initial changes during zoospore encystment, which do not require either RNA or protein synthesis, but do involve plasma membrane alterations, and 3.) the ability to regulate the encystment process by manipulating the ionic and thermal environment of the zoospore. In addition, B. emersonii is one of the most thoroughly investigated of the lower fungi, both physiologically and ultrastructurally. There is, therefore, an extensive body of literature available to provide a foundation for molecular studies. Each of these points is discussed in detail below.

1. The ultrastructural organization of the zoospore

Zoospores of B. emersonii are highly differentiated cells, about 7 x 9 μM in size, which are characterized by the lack of a cell wall and an extensive spatial segregation of their internal components. A schematic diagram of a zoospore is shown in Figure 1 (for detailed reviews see Truesdell & Cantino, 1971; Cantino & Mills, 1976). The cell does not possess an endoplasmic reticulum. Instead, the ribosomes are clustered within a single compartment, the nuclear cap, which is delimited by two parallel unit membranes (Lovett, 1963). The nuclear cap overlays a significant portion of the nucleus,

Figure 1 Schematic drawing of the zoospore of *B. emersonii* illustrating the ultrastructural organization of the cell. NCOM = nuclear cap outer membrane; NCIM = nuclear cap inner membrane; V = vacuole; M = mitochondrion; SB = SB matrix of lipid sac; BM = backing membrane; L = lipid globule; G = gamma particle; NC = nuclear cap; MT = member of microtubule triplet; NMP = nuclear membrane pore; N = nucleus; NIM = nuclear inner membrane; NOM = nuclear outer membrane; C = centriole; R = rootlet; K = kinetosome; F = flagellum; P = 'prop' connecting K to plasma membrane of spore. (Drawing taken from Myers and Cantino - see general references)

and its membranes are continuous with those enclosing the nucleus proper. Directly adjacent to the nucleus is the zoospore's single large mitochondrion. Appressed against the mitochondrion, and opposite the nucleus, is the side body complex composed of lipid globules (stored energy source) and the side body matrix. The mitochondrion, lipid globules and side body matrix are enclosed by the backing membrane which is attached to the nuclear cap membrane at numerous points (Myers & Cantino, 1974). The zoospore therefore can be considered to be comprised of an "outer membrane bag", the plasma membrane, which is directly exposed to the cell's exterior environment, and an "inner membrane bag", which encloses almost all of the cell's cytoplasmic organelles. The only major cytoplasmic organelle separate from this inner membrane system is the γ-particle, which also contains RNA, and which is intensely stained by neutral red (Myers & Cantino, 1974).

The association of almost all of the major cytoplasmic organelles within such a contiguous membrane structure suggests that it might be possible to separate these components from the plasma membrane as a single unit. In addition, the absence of an endoplasmic reticulum eliminates the most intractable of all plasma membrane preparation contaminants. Moreover, the nuclear cap and lipid globules appear as a large bright crescent and

a series of small bright bodies, respectively, with phase contrast microscopy. When the membranes enclosing these organelles are ruptured, the bright crescent disappears, and the lipid globules dissociate from the other organelles. The appearance and location of the nuclear cap and lipid bodies therefore provide an easy method for verifying the integrity of the entire membrane complex during isolation of the plasma membrane.

2. Initial changes during zoospore encystment

When zoospores are induced to encyst they proceed irreversibly through a sequence of developmental changes which results in the formation of a chitinous cell wall and the breakdown of the cell's internal organization. Ultrastructurally, the zoospore changes shape, becoming spherical with a concomitant 43% decrease in volume (Truesdell & Cantino,1971). The cell also retracts its flagellum (Cantino et al., 1968), and experiences a fragmentation of its internal membrane system, including the backing membrane/nuclear cap membrane complex (Truesdell & Cantino, 1971).

However, the first detectable changes during encystment involve alterations of the plasma membrane, specifically changes in the cell surface monitored by FITC-Conconavalin A (Jen & Haug, 1979), the induction of cell adhesiveness (Cantino et al., 1968), and the

fusion of vesicles derived from the γ-particles with the plasma membrane (Truesdell & Cantino, 1970, 1972; Myers & Cantino, 1974; Cantino & Mills, 1976).

Biochemically a number of events occur during encystment, but the most relavent to the present study are the changes observed in the cell's lipid composition. The percentage of free sterols drops, and a previously non-detectable monoglyceride appears. Moreover, there is a pronounced change in the zoospore's glycolipid fraction, with the single major glycolipid component, the diglycosyldiglycerides, decreasing by 55% during encystment (Mills et al., 1974).

Physiologically membrane fusion processes are of crucial importance in zoospore encystment, since it has been demonstrated that the last enzyme of the biosynthetic pathway for chitin formation, chitin synthetase (EC 2.4.1.16), is located in the γ-particles (Myers & Cantino, 1974). Deposition of a cell wall has been correlated to the fusion of γ-particle derived vesicles with the plasma membrane (Cantino & Myers, 1973; Myers & Cantino, 1974). In addition chemical analysis of the γ-particle indicates that a high percentage of the lipids present are glycolipids, especially diglycosyldiglycerides (Mills & Cantino, 1978).

It is also significant that the initial changes which occur during encystment do not require either

protein or RNA synthesis (Lovett, 1968, 1975; Soll & Sonneborn, 1971 a, b; Silverman et al., 1974). As a consequence, the molecular events associated with zoospore encystment must involve a re-organization of pre-existing components. The role(s) of the plasma membrane's physical-chemical properties in these events can therefore be examined independently.

3. Environmental control of zoospore encystment

Cations and temperature play an important role(s) in the regulation of the encystment process. Potassium ion addition has been shown to induce zoospore encystment. This induction process was found to be temperature dependent, with a dramatic change in the kinetics of zoospore encystment occurring between 10 and 15°C. At 15°C or above zoospores encysted within minutes, whereas at 10°C or lower it took hours, it it occurred at all (Soll & Sonneborn, 1969).

In contrast, Ca^{2+} ions (but not Mg^{2+} ions) can prevent encystment (Cantino, et al., 1968; Soll & Sonneborn, 1969, 1972). In the presence of millimolar Ca^{2+} ion concentrations (K^{+} ions not added) zoospores did not encyst at their growth temperature, i.e., 20°C (Soll & Sonneborn, 1972; Suberkropp & Cantino, 1972). However, zoospores did encyst if the temperature was raised to 27°C, even in the presence of much higher Ca^{2+} ion concentrations (Soll & Sonneborn, 1972). These results suggest an upper limit for the Ca^{2+} ion effect, which can be overcome at higher temperatures.

In addition to the effects of increased temperature, the effect of Ca^{++} ions on zoospore encystment can be reversed by the addition of K^{+} ions to the cell suspension. Moreover, zoospores preloaded with $^{45}Ca^{++}$ have been reported to release the $^{45}Ca^{++}$ ions immediately upon the inclusion of K^{+} ions (Soll & Sonneborn, 1972).

Temperature limits for zoospore viability have also been described. At $4^{o}C$ or less zoospore respiration goes to zero, and at $0\text{-}1^{o}C$ the cells lyse rather than encyst (Cantino et al., 1969; Truesdell & Cantino, 1972; Suberkropp & Cantino,1972). At $37^{o}C$ zoospores will encyst and develop normally, but no development occurs at $39^{o}C$ (Soll & Sonneborn, 1969).

Together these results suggest a relationship between temperature, calcium ions and potassium ions on zoospore differentiation. Since the initial changes during zoospore encystment involve the plasma membrane, and temperature and cations have been shown to be the two main factors altering the physical-chemical properties of membranes, it is reasonable to hypothesize that the dynamic properties of the plasma membrane are involved in the regulation of zoospore differentiation.

This dissertation is divided into four sections. The purpose of the first section is to establish a correlation between the physiological effects of temperature and cations on zoospore differentiation and their effects

on the physical-chemical properties of the zoospore plasma membrane in vivo. The second section expands upon the first and examines the roles of the various lipid fractions of the zoospore in these phenomenon. The third section describes a procedure for the isolation of the zoospore plasma membranes, and their analysis in order to clarify any relationship between the first two sections. The fourth section specifically examines one aspect of the first section, the middle break point, T_M, to determine if it is the result of a lipid/lipid or lipid/protein interaction and to ascertain the effects of Ca^{++} and K^{+} ions on this interaction.

The analytical method employed for the bulk of these studies is electron spin resonance spectroscopy (ESR). This method was chosen because of its extreme sensitivity to subtle changes in the physical-chemical properties of a membrane, and its ability to determine the dynamic properties of such a membrane.

SECTION I

EFFECTS OF CATIONS ON THE PLASMA MEMBRANE OF *BLASTOCLADIELLA EMERSONII* ZOOSPORES. AN ESR STUDY.

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Key Words: Membranes, Cations, Differentiation, *Blastocladiella emersonii*

Summary

The physical properties of the plasma membrane of the aquatic Chytridiomycete Blastocladiella emersonii were investigated, in particular the effects of cations on membrane structure. Intact zoospores and lipid extracts were labelled with the spin-labels 5-nitroxystearate (5-NS), 12-nitroxystearate (12-NS), and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO). Electron spin resonance spectroscopy indicated a total of three breaks in plots of the hyperfine splitting parameter, $2T_{||}$, order parameter, S, and the partition coefficient, f, vs. temperature. The first and third break points (T_L and T_H) were found to be independent of the external K^+, Ca^{++}, or Mg^{++} ion concentrations. They were similar to the break points found in aqueous dispersions of lipid extracts and correlate well with the temperature limits for zoospore viability. In contrast, the middle break point (T_M) was markedly influenced by the external Ca^{++} ion concentration. Ca^{++} ions increased T_M from 12°C (no Ca^{++} added) to 22°C (10 mM Ca^{++}), i.e., growth temperature. K^+ ions reversed this Ca^{++} effect, downshifting T_M from 22°C to 10°C. A comparison of the physico-chemical effects of these ions on the membrane, as revealed by the cation-induced shift in T_M is closely correlated with the temperature dependence and physiological effects of cations on zoospore differentiation. This suggests that cations may modify the physical state of the

plasma membrane and be involved in regulating the initial changes during zoospore encystment.

Introduction

Zoospores of the aquatic chytridiomycete Blastocladiella emersonii are highly differentiated cells which can undergo rapid morphological changes in response to their environment (1-5). Prior to encystment and germination, the motile zoospore is characterized by the absence of a cell wall and an extensive spatial segregation of its internal components (6,7). When zoospores are induced to encyst they proceed irreversibly through a sequence of developmental changes which results in the formation of a chitinous cell wall and the breakdown of their internal organization (5,7,8). The encystment process occurs rapidly and seems to require neither protein nor RNA synthesis (9-14). However, the first detectable changes during encystment do involve alterations of the plasma membrane, specifically changes in cell surface fluorescence properties (15), the induction of cell adhesiveness (6), and the fusion of vesicles derived from the γ-particles with the plasma membrane (16,17).

Ions and temperature play an important role(s) in the regulation of the encystment process. Physiological studies on B. emersonii have demonstrated that K^+ ions can trigger encystment, and that this induction is temperature dependent (2). In contrast, Ca^{++} ions (but not Mg^{++}) can prevent encystment (2,3,6). This Ca^{++} ion effect can be

reversed by increasing the temperature, or including K^+ ions in the buffer (3). Cations (especially Ca^{++}) and temperature are known to affect the physical properties of model and biomembranes, including many membrane associated functions (18-24). Since the initial changes during encystment involve the plasma membrane, it is reasonable to hypothesize that at least part of the effects of ions on zoospore differentiation involve changes in the physical properties of this membrane. To test this hypothesis we monitored the state of the zoospore plasma membrane, in vivo, with ESR spectroscopy, as a function of temperature and cation concentration using the spin-labels 12-nitroxystearate (12-NS), 5-nitroxystearate (5-NS), and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO). The information presented in this paper indicates that cations do affect the dynamic state of the plasma membrane, in vivo, in a way which correlates very well with the previously reported effects of ions on zoospore differentiation.

Materials and Methods

Organism, Medium and Growth

Blastocladiella emersonii was kindly supplied by Dr. E. C. Cantino (Department of Botany and Plant Pathology, Michigan State University). The organism was routinely grown on PYG agar at 22°C as previously described (25). Zoospores were harvested from first generation plants by flooding each plate with a buffered (5 mM Morpholinopropane sulfonic acid (MOPS), final pH 6.6 - 6.8) solution

containing the particular Ca^{++} or Mg^{++} ion concentration being tested (K^{+} ions, final concentration 50 mM, were added after resuspension of the pellet to avoid encystment during harvesting). The zoospore suspension was then collected, filtered to remove germlings and plants, and gently pelleted by centrifugation (1000 x g for 5 min at room temperature).

Spin-Labeling Procedure

Pellets were resuspended, 0.5 ml samples removed (4.0 to 5.0 x 10^9 cells) and labelled with an ethanolic solution of 12-NS, 5-NS, or TEMPO (conc. approx. 1 molecule spin-label/6000 molecules of lipid). Just prior to transfer of the sample into the ESR cuvette, $K_3Fe(CN)_6$ was added (final conc. 1 mM) to prevent disappearance of the spin-label. Control experiments (not shown) indicated that neither the concentration of ethanol (approx. 1%) nor $K_3Fe(CN)_6$ used altered the ESR spectra recorded. The ethanol concentration used was also shown not to affect cell viability. In some experiments ethylene glycol (final conc. 33%) was added to the sample just before transfer into the ESR cuvette to depress the freezing point. Control experiments demonstrated that though there was an increase in the hyperfine splitting parameter ($2T_{||}$) values obtained, there was less than a one degree shift in the temperature at which break points were found in plots of $2T_{||}$ vs.temperature.

Lipid Extraction and Vesicle Preparation

Lipids were isolated from zoospores as previously reported (26). Aqueous dispersions were prepared from the total lipid extract by drying an aliquot of the mixture (30-40 mg dry wt.) first under N_2, and then under vacuum. The sample was resuspended on a vortex stirrer into 0.5 ml buffer (pH 6.7) with a glass bead. The suspension was then sonicated (125 watt sonic water bath), spin-label was added, and the suspension was again sonicated. The concentration of spin-label was less than 0.2% wt./wt.

Spin-Label Measurements and Analysis

ESR measurements were made with a Varian X-band spectrometer (Model E-112). The temperature was regulated with a Varian variable temperature controller and monitored inside the cuvette with a calibrated thermocouple which was connected to a digital readout meter (Omega model 250). All spectra were recorded at power and modulation amplitude settings which were previously determined to be below those causing saturation or line width broadening. The preferential incorporation of the spin-label into the plasma membrane was ascertained by ascorbate reduction of the spin-probe (27), and protection of the spin-label from reduction using $K_3Fe(CN)_6$ or $Na_3Fe(CN)_6$ (28).

The spectra were analyzed with a Varian 620/L-100 computer. The order parameter (S) was calculated according to method number two of Jost and Griffith (29). The

partition parameter (f) was determined according to the method of Shimshick and McConnell (30). The experimental plots (hyperfine splitting parameter $2T_{||}$, S, or f vs. temperature) were analyzed using two methods. The first method was in terms of linear components by fitting regression lines to appropriate sections using the method of least squares. This method of analysis has been frequently used in the interpretation of results obtained from ESR experiments,and the temperatures at which break points are observed (indicated by the intersections of straight lines) are in good agreement with those obtained by other physical-chemical techniques (22,30,31). This method also included the determination of the correlation coefficient, r, a measure of the goodness of fit, which indicated whether the initial assumption of a stright line was valid. An r value of 1.0 indicates a perfect fit between the data points and the calculated line. In all cases $r > 0.96$ with most lines having $r \geq 0.98$. The second method of analysis was in terms of an iterative least squares program (Brunder, Coughlin, and McGroarty, unpublished) using normalized β-splines developed by Dierckx (32). This method involved the simultaneous analysis of $2T_{||}$, S, or f over the entire temperature range, and determined the temperatures at which break points occurred. Both methods of analysis gave the same results. The spectra obtained with 12-NS, however, could not be

analyzed in this fashion due to the absence of a high field trough even at 0°C which made measurements of $2T_{||}$ impossible. Use of this spin-label was therefore discontinued.

Results

Representative spectra of 5-NS labeled zoospores recorded at different temperatures are shown in Figure 1. The spectra obtained, especially at higher temperatures, are similar to those observed for spin-labeled sarcoplasmic reticulum, spinach chloroplasts, yeast cells (18), erythrocytes (33), and E. coli membranes (34). These previously published spectra were interpreted as indicating the presence of at least two superimposed spectral components, one corresponding to a relatively immobilized spin-label population, and another to a relatively mobile population, here associated with peaks A and B. To gain further insight into the spectral shape changes observed, the heights of the two low field peaks A and B, were measured at different temperatures. The ratio of the two peak heights (H_A/H_B) was found to change as a function of temperature (Table I). This change is similar to that recently observed for spin-labeled E. coli membranes, where changes in the (H_A/H_B) ratio were measured as a function of divalent cation concentration (34). In B. emersonii zoospores the spectral contribution of peak B increased with increasing temperature. However, there

Figure 1 Representative ESR spectra of 5-nitroxy-stearate labelled B. emersonii zoospores recorded at different temperatures. The ratio of the two low field peak heights (H_A/H_B) changed reversibly as a function of temperature. ———, 2°C; ·····, 9°C; -------, 36°C.

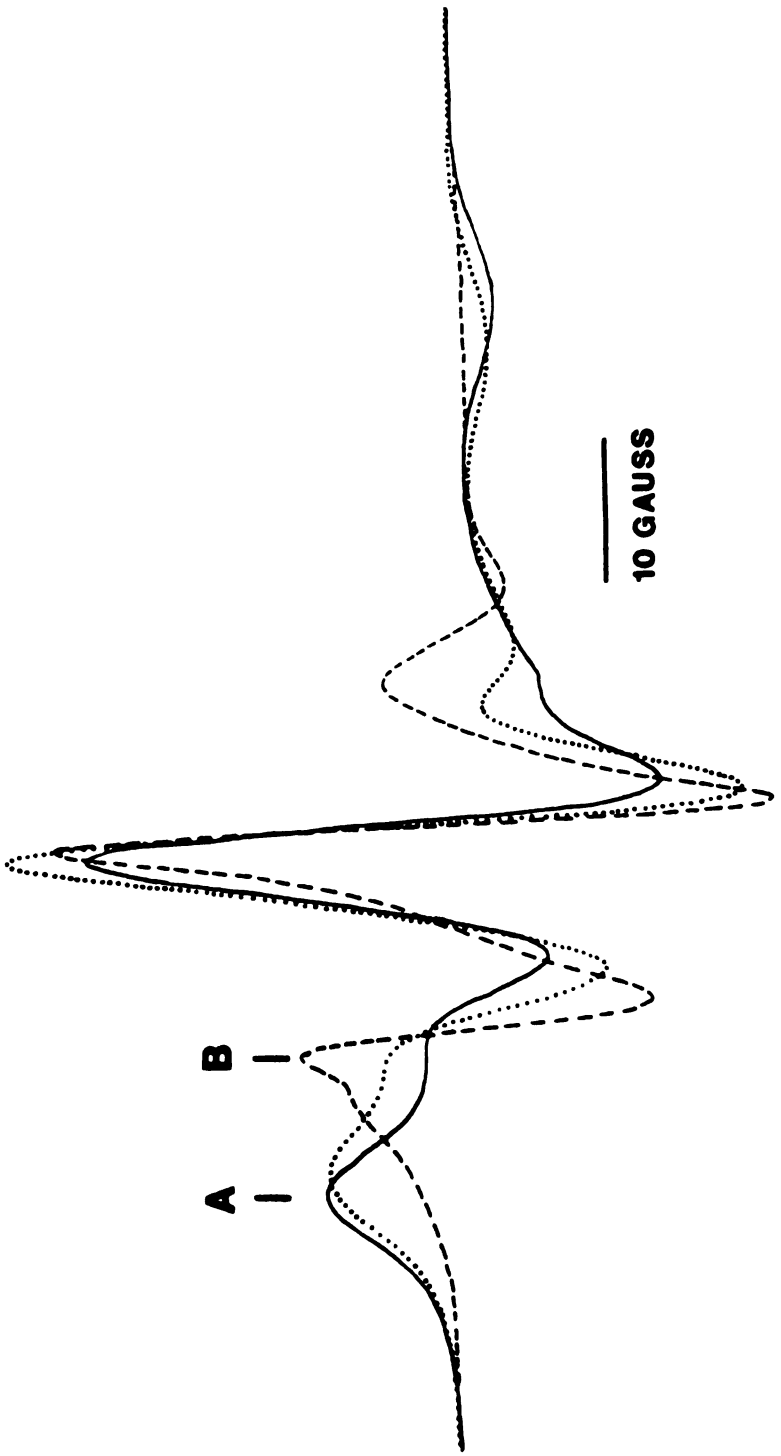

10 GAUSS
B
A

Table I. Ratio of the heights of the low field peaks (H_A/H_B) observed in ESR spectra and measured at different temperatures. B. emersonii zoospores spin-labeled with 5-nitroxystearate (5-NS). Values are averages of four experiments.

	Temperature °C					
	0°	5°	10°	15°	20°	25°
Ratio H_A/H_B	3.54	2.38	1.62	1.24	0.97	0.84
S. D.	±0.22	±0.18	±0.13	±0.07	±0.048	±0.059

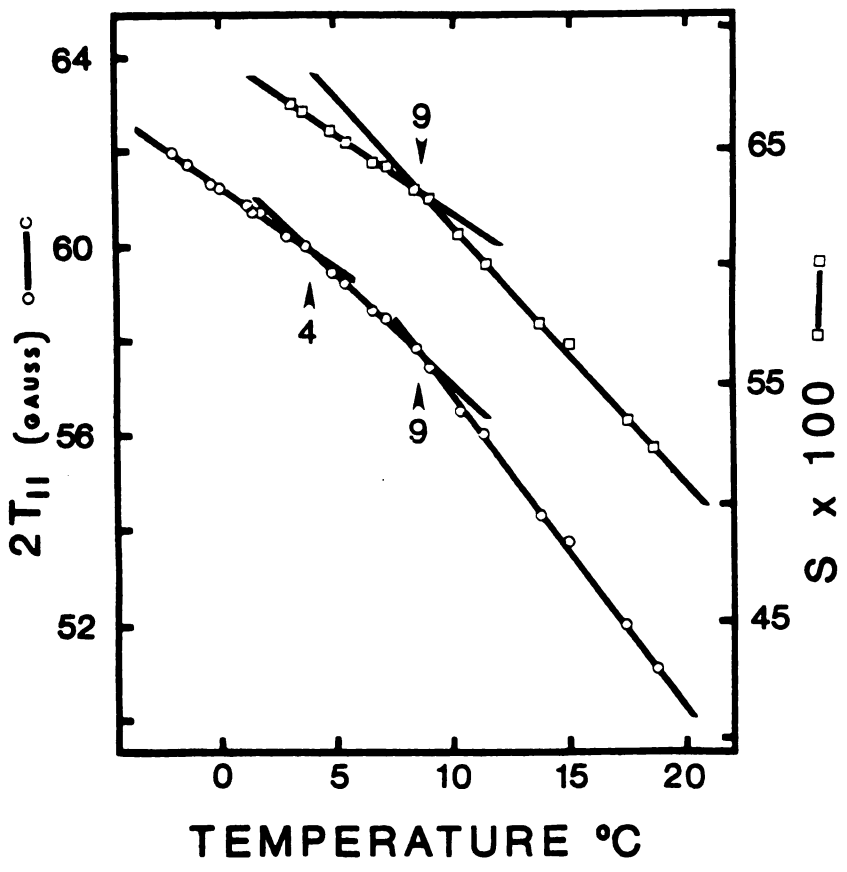
was little, if any, change in its position. In contrast, the spectral contribution of peak A changed both in relative peak height and position as a function of temperature (Figure 1). Addition of ascorbate to reduce the spin-label resulted in the simultaneous and total disappearance of both low field peaks (data not shown).

The effects of ion addition on the plasma membrane of intact zoospores labelled with 5-NS were evaluated by measuring the hyperfine splitting parameter, $2T_{||}$, and where possible, the order parameter, S. The hyperfine splitting value, $2T_{||}$, is highly sensitive to changes in the molecular environment of the spin-label (35), and is related to the freedom of motion of the spin-label in the membrane, lower $2T_{||}$ values being associated with a greater freedom of motion of the probe (36,37). The order parameter, S, is dependent on $2T_{\perp}$ as well as $2T_{||}$. Various models have been proposed for the interpretation of the order parameter at the molecular level, including models based upon molecular motion (29), and others based upon molecular orientation (38). In either case a large value for S (up to 1.0) indicates a high degree of order and a small value for S (down to 0) indicates a low degree of order. Plots of these two parameters vs. temperature have been used extensively to determine changes in the physical properties of both model and natural biomembranes (22,24, 31). To eliminate possible artifacts due to overlap of

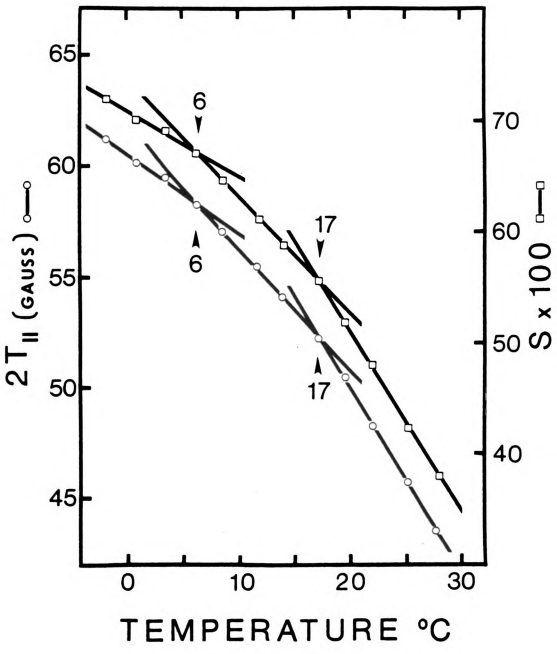
the two low field peaks $2T_{||}$ and S were only measured at temperatures where the two low field peaks of the spectrum were clearly resolvable (up to 26-28°C). By restricting the measurement of $2T_{||}$ to this temperature range and observing the spectra obtained at low temperatures, where the spectral contribution of peak B was minimized, it was possible to attribute changes in $2T_{||}$ to changes in peak A. Figure 2 illustrates the effects of various ions and ion concentrations on $2T_{||}$ and S as a function of temperature. In all cases two break points were observed. Plots of $2T_{||}$ and S exhibited the same break point temperatures. The results obtained in the presence of buffer only, buffer + 10 mM $MgCl_2$, or buffer + 50 mM KCl were very similar (Figure 2a). The first break point occurred at 3-4°C in buffer + 50 mM KCl, or at 4-6°C in buffer only or buffer + 10 mM $MgCl_2$. The second break point varied only slightly, occurring at 8-9°C in buffer + 50 mM KCl, at 10-12°C in buffer only, or at 11-13°C in buffer + 10 mM $MgCl_2$. Ca^{++} ions did not significantly alter the temperature at which the first break occurred (5-7°C), but had a marked affect on the temperature at which the second break point, T_M, was observed (Plots 2b,c). In the presence of 1 mM Ca^{++} this break point was shifted from 10-12°C to 17-18°C. Increasing the Ca^{++} ion concentration to 5 mM Ca^{++} shifted T_M to 19-20°C, and 10 mM Ca^{++} further shifted the break point to 20-22°C. 20 mM Ca^{++} did not further change the

Figure 2 Plots of $2T_{||}$ and S vs. temperature for B. emersonii zoospores labelled with 5-nitroxystearate showing the effects of ions on the position of the break points. Two breaks for $2T_{||}$ were found in all cases. S was calculated where possible. Arrows indicate break points. Note the agreement between the break points determined with $2T_{||}$ and those obtained with S (pH 6.6 to 6.7).

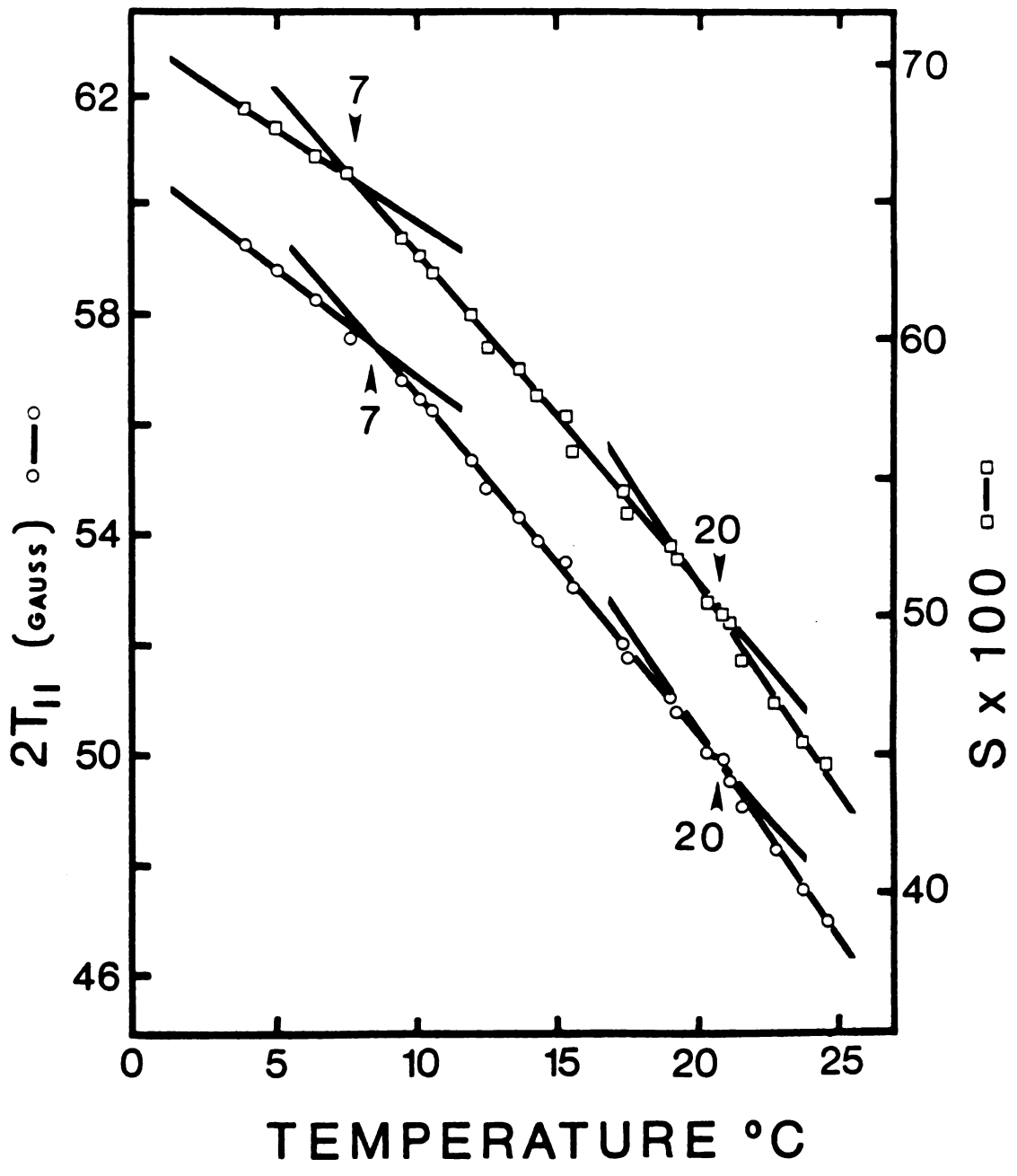
2a. Zoospores in the presence of 50 mM KCl. Break points were very similar to those determined in the presence of buffer only or 10 mM $MgCl_2$. $2T_{||}$,O—O; S,□—□.


64
60
56
52
65
55
45
9
4
9
0
5
10
15
20
2T∥ (GAUSS) ○—○
S x 100 □—□
TEMPERATURE °C

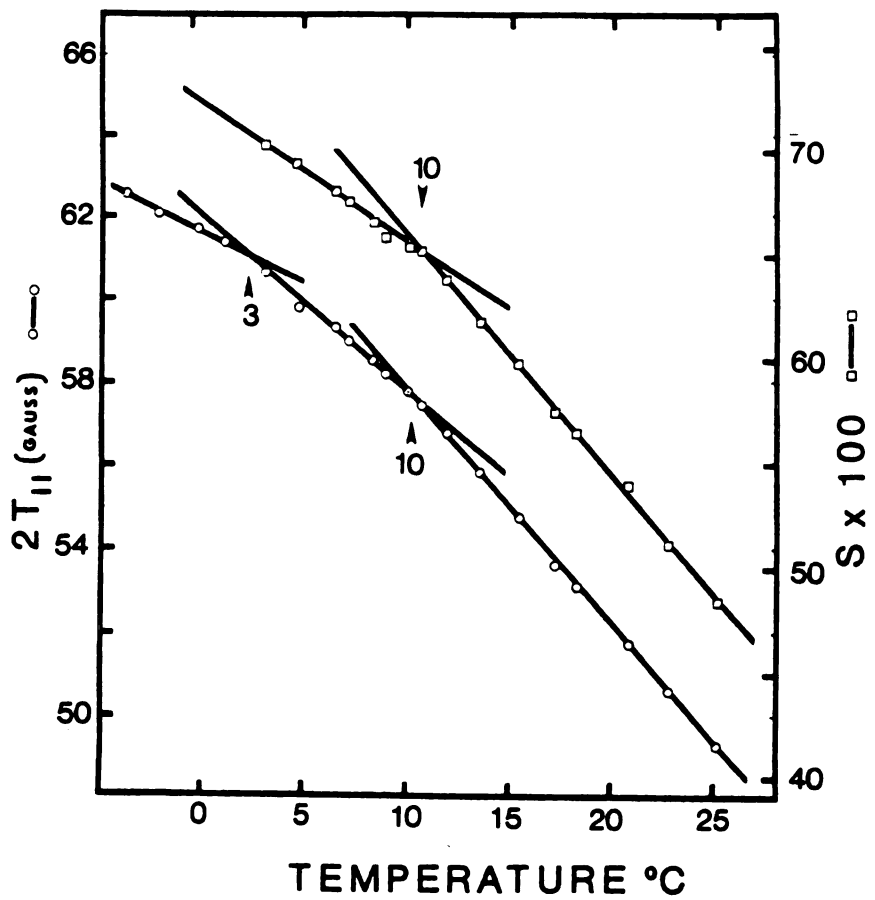
2b. Zoospores harvested in the presence of 1 mM $CaCl_2$. $2T_{||}$,O—O; S,□—□. Note increase in the temperature at which the second break occurred.


65
60
55
50
45
70
60
50
40
0
10
20
30
6
6
17
17
2 T_II (GAUSS)
S x 100
TEMPERATURE °C

2c. Zoospores harvested in the presence of 10 mM $CaCl_2$. Break points were the same as those obtained in the presence of 20 mM $CaCl_2$. $2T_{||}$, O—O; S, □—□ .


62
58
54
50
46
70
60
50
40
0
5
10
15
20
25
7
7
20
20
2T⊥ (GAUSS) ○—○
S x 100 □—□
TEMPERATURE °C

2d. Reversibility of the Ca^{++} effect by K^{+} ions. Zoospores were harvested as in experiments represented in Figure 2c. After spin-labeling, but before transfer to the ESR cuvette, KCl was added (final conc. 50 mM KCl). $2T_{||}$,O—O; S,□—□ .

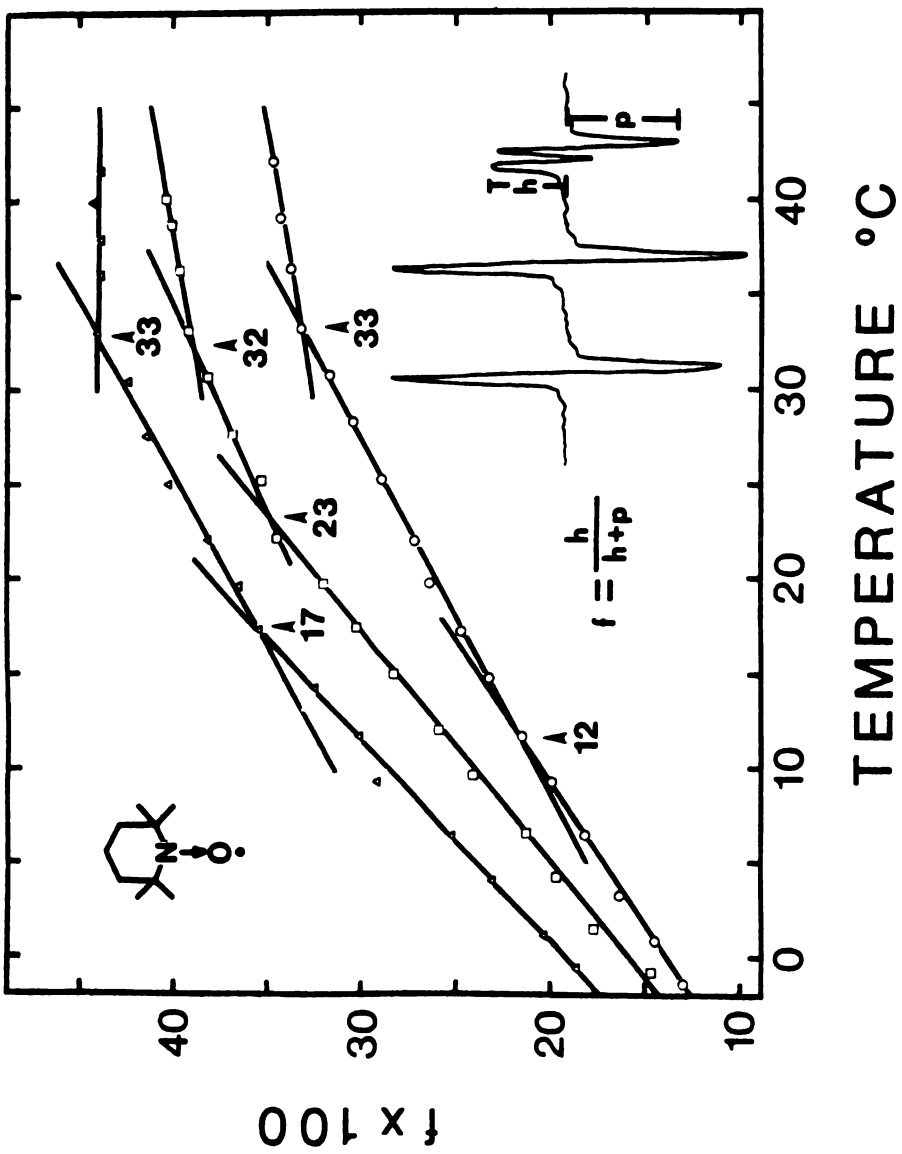

66
62
58
54
50
70
60
50
40
0
5
10
15
20
25
10
3
10
2 T|| (GAUSS)
S x 100
TEMPERATURE °C

temperature at which the second break point occurred, suggesting a possible saturation effect. The Ca^{++} ion effect observed could be reversed by K^{+} ions (Figure 2d). When KCl was added (50 mM final conc.) to samples already in the presence of 10 mM Ca^{++}, the second break point T_M was shifted back to 10-11°C. To ascertain whether any break points occurred below 0°C, 5-NS labeled zoospores were studied in the presence of ethylene glycol. No additional break points were detected between -12°C and 0°C, nor were the positions of the break points above 0°C significantly altered (data not shown).

To determine whether there were any break points at higher temperatures (>25°C) which could not be observed with 5-NS, we used the spin-label TEMPO. TEMPO is a molecule which has been shown to be soluble in water and in fluid, liquid-crystalline membranes, but not in bilayers in the gel state(29,30,39). The spectral parameter, f, is related to the fraction of the membrane which is accessible to the spin probe. Since f is determined by the partitioning of the spin-label between the aqueous phase and the fluid membrane, TEMPO not only allowed us to measure the physical behavior of the zoospore membrane at higher temperatures, but also provided a different method of verifying the results obtained with 5-NS.

Figure 3 illustrates the data obtained for zoospores labelled with TEMPO in the presence of different Ca^{++} ion

Figure 3 Plot of f x 100 vs. temperature for B.emersonii zoospores labelled with TEMPO (pH 6.6 to 6.7). f was calculated as indicated since the line widths for both H and P were found to be equal. Zoospores harvested in buffer only, ○ —— ○; 1 mM $CaCl_2$, △ —— △ ; 10 mM $CaCl_2$, □ —— □ . Plots of 10 mM $MgCl_2$ were the same as buffer only.


f x 100
40
30
20
10
0
10
20
30
40
TEMPERATURE °C
33
32
33
23
17
12
f = h/(h+p)
I_h
I_p

concentrations as a function of temperature. In these experiments it was not possible to determine the absolute amount of membrane lipid in the liquid-crystalline state, due to the variable number of cells present. However, changes in the partitioning of the spin-label measured in the same sample as a function of temperature do reflect changes in the relative fraction of the zoospore plasma membrane which was accessible to the spin-label. In all cases, an upper break point, T_H, was observed between 32 and 34°C which was independent of Ca^{++} ion concentrations. In contrast to this upper break point, another break occurred at lower temperatures which was influenced by the Ca^{++} ion concentration. For samples in the presence of 10 mM $CaCl_2$ this break occurred at 22-23°C. Reducing the Ca^{++} ion concentration to 1 mM $CaCl_2$ shifted this break point down to 17-18°C. Zoospores in the presence of buffer only, or buffer + 10 mM $MgCl_2$ were characterized by a shallow break which occurred at 10-12°C. Thus, the results derived from the TEMPO experiments confirm those obtained with 5-NS for the Ca^{++} ion dependent break point, T_M. These results are summarized in Table II. Breaks were not detected at 4-6°C using the spin-label TEMPO. However, no firm conclusions can be drawn since it was not possible to determine f accurately below 0°C.

To examine the role(s) of lipids in these observed phenomena, zoospore lipids were isolated, aqueous

Figure 4 Plot of $2T_{||}$ vs. temperature for aqueous dispersions of B. emersonii zoospore lipid extract labelled with 5-nitroxystearate.
O, sample run in the presence of buffered ethylene glycol (final conc. 33%).
□, sample run in the presence of buffer only.
Note: the ethylene glycol did affect the absolute value of $2T_{||}$ observed and the data presented were normalized to those obtained with buffer only. Break points were shifted less than one degree in the presence of ethylene glycol.

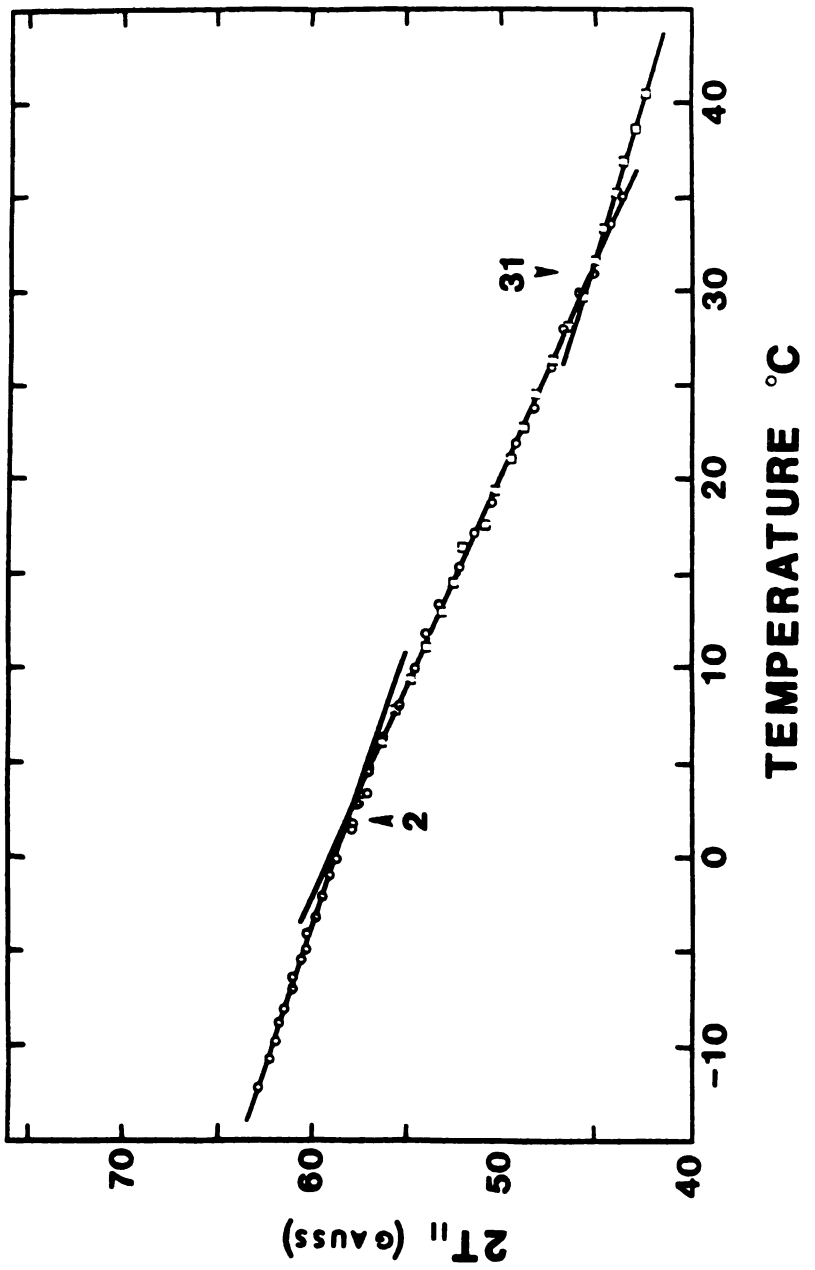

2T⊥ (gauss)
70
60
50
40
TEMPERATURE °C
-10
0
10
20
30
40
2
31

Table II. Thermal transformation points of membranes of *B. emersonii* as a function of ion concentration

Spin-label		Break point		
		Lower T_L (°C)	Middle T_M (°C)	Higher T_H (°C)
5-NS	Buffer only*	4-6	10-12	
	+ 10 mM $MgCl_2$	4-6	11-13	
	+ 50 mM KCl	3-4	8- 9	
	+ 1 mM $CaCl_2$	5-7	17-18	
	+ 10 mM $CaCl_2$	5-7	19-20	
	+ 20 mM $CaCl_2$	5-7	20-22	
	+ 10 mM $CaCl_2$ + 50 mM KCl	2-3	10-11	
Tempo	Buffer only		10-12	32-34
	+ 1 mM $CaCl_2$		17-18	33-34
	+ 10 mM $CaCl_2$		22-23	33-34
	+ 10 mM $MgCl_2$		10-12	33-34
5-NS labeled lipid extract		2-4		30-32
	Buffer only			

*Buffer: 5 mM Mops, pH 6.7

dispersions were labelled with 5-NS, and the temperature dependence of the hyperfine splitting parameter, $2T_{||}$, was determined over the temperature range -12°C to 42°C. A plot of $2T_{||}$ as a function of temperature is shown in Figure 4. Two break points were observed, one at 2-4°C and the other at 30-32°C. These values are very similar to the lower and upper break points, T_L and T_H, observed in whole cells with 5-NS and TEMPO respectively (Table II). Ca^{++} ion addition did not affect the position of these break points. However, it did increase the $2T_{||}$ values obtained (by 5-7 gauss) over the entire temperature range. In addition, no breaks were detected between these two, in the presence or absence of Ca^{++} ions. Since the H_A/H_B ratios were significantly different from those obtained with zoospores labelled with 5-NS, there are probably differences in the relative amounts and organization of the extracted lipids as compared to the plasma membrane of the zoospore. Such differences would explain the absence of the middle, ion dependent break point, T_M.

Discussion

The data presented in this paper support two conclusions. (1) The temperature limits of zoospore viability are related to the phase transformation temperatures of the lipid matrix. If T_L and T_H represent the onset and completion of a gel-to-liquid-crystalline phase transition, respectively, zoospore viability is

related to a mixed lipid state. (2) Specific ions markedly affect the physical properties of the plasma membrane, which in turn are related to the physiological effects of these ions on zoospore differentiation.

The hyperfine splitting parameter ($2T_{||}$) values obtained for 5-NS labelled zoospores indicate that the plasma membrane is much more fluid at its growth temperature than the plasma membrane of organisms such as T. acidophila (22), or E. coli (31). Initial experiments utilizing 12-NS also suggest a very fluid microenvironment. This observation is consistent with preliminary findings in our laboratory showing that the isolated plasma membrane contains a large percentage of unsaturated fatty acyl chains, including arachidonic acid (20:4). Similar levels of unsaturated lipids have also been reported in B. emersonii zoospore lipid extracts (26).

Analysis of the data obtained from spin-labelled zoospores indicated three break points in plots of spectral parameters ($2T_{||}$, S, f) vs. temperature (Table II). Of these three, the lower and upper transition points, T_L and T_H, were found to be independent of ion concentration. They were also very similar to the two break points found in 5-NS labelled aqueous dispersions of whole cell lipid extracts. Both intact cells and lipid extracts were characterized by the same transformation temperature range ΔT of about 28°C, where $\Delta T = T_H - T_L$. In lipid extracts

T_L and T_H were shifted to lower temperatures by approximately three degrees as compared to the break points determined in spin-labeled zoospores. Such a downshift of T_L and T_H probably reflects the absence of proteins in the lipid extract dispersions, or organizational differences in the two membranes.

Break points observed with ESR spectroscopy in multicomponent systems have been correlated with lipid phase transitions, lateral phase separations, lipid clusters or lipid/protein interactions (30,39,40-43). In our experiments both protein-free lipid extracts and intact zoospores had similar break points (Table II). In addition, the partition parameter, f, only increased slightly above T_H (Figure 3). This has previously been interpreted to indicate the presence of a fluid phase membrane (29,30,39). The temperatures, T_L and T_H, may therefore represent the onset and end of a gel-to-liquid-crystalline phase transformation of the lipid environment associated with spectral component A.

The lower and upper phase transformation temperatures, T_L and T_H, correlate well with the temperature range of zoospore viability. Previous studies indicated a lower temperature viability limit of 1-4^{o}C (5), and an upper growth temperature limit of 37-39^{o}C. We confirmed these results finding temperature limits of 1-3^{o}C and 34-36^{o}C (data not shown). These observations suggest that the

temperature range over which the zoospore remains viable is correlated with that where plasma membrane lipids exist in a mixed state. The necessity of a mixed lipid state for cell viability is in accordance with findings for E. coli outer membranes (31).

In contrast to the lower and upper break points, T_L and T_H, the temperature at which the middle break point occurred (T_M) was markedly influenced by the ionic environment (Figures 2 and 3, Table II). The reference value determined for T_M in buffer alone was about 12°C. Addition of either K^+ ions or Mg^{++} ions changed this value only slightly. However, increasing the external Ca^{++} ion concentration progressively shifted the T_M value in both 5-NS and TEMPO labelled cells up to about 22°C, i.e., around the growth temperature, where this effect saturated. These results suggest that the shift of T_M upwards was Ca^{++} ion selective rather than an electrostatic divalent cation effect. Furthermore, ionic strength could not account for this shift since the concentrations of K^+ ions employed were much greater than those of Ca^{++} ions.

A comparison of these ionic effects on T_M with the temperature dependence and physiological effects of the same ions on zoospore differentiation indicated a high degree of similarity. A dramatic change in zoospore encystment kinetics was found to occur between 10°C and 15°C in the presence of K^+ ions (2). At 15°C or above

zoospores encysted within minutes, whereas at 10°C or lower it took hours if it occurred at all.

In the presence of millimolar Ca^{++} ion concentrations (K^+ ions not added), zoospores did not encyst at their growth temperature, i.e., 20°C (3,4). However, zoospores did encyst if the temperature was raised to 27°C, even in the presence of much higher Ca^{++} ion concentrations (3). These results indicate that there is an upper limit for the Ca^{++} ion effect, which lies between 20 and 27°C. This is consistent with our experiments which demonstrated an upper limit for T_M at about 22°C as a function of Ca^{++} ion concentration. In addition, Ca^{++} ions were found to be more effective than Mg^{++} ions in preventing encystment, indicating that the inhibition was relatively Ca^{++} selective (5). Similar effects of these ions on T_M are shown in Table II.

Physiological experiments indicate that K^+ ions can reverse the effect of Ca^{++} ions on zoospore encystment (3). This implies that a similar effect should be observed physico-chemically for T_M, if the two ion effects are indeed related. The results presented in Figure 2 (c,d) demonstrate that K^+ ions do reverse the effects of Ca^{++} ions on the plasma membrane. This hypothesis is in accord with findings that $^{45}Ca^{++}$ ions were immediately released from preloaded zoospores upon K^+ addition (3).

The molecular nature of the phenomena responsible

for the middle break point and the ion-induced shifts in T_M remains unknown. Since both TEMPO and 5-NS were sensitive to these shifts in T_M, the membrane lipids are certainly involved. In addition, ESR experiments with the isolated zoospore plasma membrane, spin-labeled with 5-NS, also show a distinct middle break point (T_M) at 23-25°C in the presence of 10 mM $CaCl_2$ in plots of $2T_{||}$ vs. temperature, measured over the temperature range 0-40°C (Leonards and Haug, unpublished). It is noteworthy, however, that irrespective of the position of T_M, the $2T_{||}$ and S values observed remained relatively unchanged as determined by the 5-NS spin probe (Figure 2). In model systems addition of Ca^{++} to negatively charged phospholipids resulted in an increase in $2T_{||}$ and S as well as a shift in the transition point (18,40,44). This suggests that the actual fluidity of the bulk phase lipids in the zoospore membrane may not be affected by Ca^{++} ions. Instead, the Ca^{++} and K^+ ions may be acting more specifically at the cell surface, perhaps by altering lipid/protein interactions, lipid head-group orientations, or the clustering of membrane components. These interactions may be similar to the surface antagonism of Na^+ and Mg^{++} observed in chloroplast grana thylakoid membranes (45). Such interactions would be consistent with the observation that addition of K^+ ions alters the fluorescence properties of the cell surface within 15 seconds (15). These

possibilities are now being investigated.

Acknowledgements

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SECTION II

ELECTRON SPIN RESONANCE STUDY OF THE ISOLATED LIPID COMPONENTS FROM *BLASTOCLADIELLA EMERSONII* ZOOSPORES

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Key Words: Lipid Dispersions, ESR, Glycolipids, Cation Effects, Phase Transitions, *Blastocladiella emersonii*, Zoospores

Abbreviations: MOPS, Morpholinopropane sulfonic acid; TLC, Thin-layer chromatography; GLC, Gas-liquid chromatography; 5-NS or 5-nitroxystearate, {2-(3-Carboxypropyl)4, 4-dimethyl-2-tridecyl-3-oxazolidinyloxyl}

SUMMARY

The physical-chemical properties of lipid components isolated from zoospores of the aquatic phycomycete Blastocladiella emersonii were investigated with electron spin resonance (ESR) spectroscopy using the spin label 5-nitroxystearate. Lipid dispersions were made from zoospore phospholipids and glycolipids, both singly and in combination with each other and with isolated neutral lipid components.

Plots of the hyperfine splitting parameter ($2T_{||}$) vs. temperature indicate that it is the zoospore glycolipids rather than the phospholipids which are responsible for the phase transformations previously observed in aqueous dispersions of the total lipids extracted from zoospores and in zoospores in vivo. The discontinuities observed in the glycolipid dispersions seem to represent the onset and completion of a gel-to-liquid-crystalline phase transition. Over the temperature range tested, Ca^{2+} increased the rigidity of the glycolipid dispersions, the major component of which is probably a diglucosyldiglyceride, but had no effect on the phospholipid dispersions. This increase in $2T_{||}$ was not affected by inclusion of neutral lipids into the glycolipid dispersion but was eliminated at high (5 to 1 wt./wt.) phospholipid to glycolipid ratios. The Ca^{2+} effect was relatively independent of both the absolute rigidity

of the dispersion and its phase (gel or liquid-crystalline), suggesting an interaction with the glycolipid head group rather than the hydrocarbon core. The Ca^{2+} ion-induced increase in $2T_{||}$ was neither prevented nor reversed by the presence of K^{+} ions.

The presence of two spin label populations co-existing in a dynamic equilibrium was found in glycolipid/neutral lipid dispersions. Plots of the percentage ($\{H_A/(H_A + H_B)\} \times 100$) of the spin label population, as measured by the peak height of the low field peaks, corresponding to the more immobilized component (H_A) vs. temperature indicated two break points. The temperatures at which these break points occurred are similar to those obtained for the glycolipid dispersions, and match the break points (T_L and T_H) found in ESR experiments using zoospores <u>in vivo</u>.

The importance of the glycolipids in the development of this organism is discussed.

INTRODUCTION

The Chytridiomycete Blastocladiella emersonii is an excellent model system for studying the role(s) of membranes in cellular differentiation. The zoospores of this organism are highly differentiated cells which can be triggered to undergo rapid and irreversible morphological changes (1-4). These developmental changes do not seem to require either protein or RNA synthesis (5,6), but do involve extensive membrane alterations. The membrane changes include the onset of cell adhesiveness (7), the fusion of cytoplasmic vesicles with the plasma membrane (8,9), and the appearance of a chitinous cell wall (4-6).

In a previous study, we established a correlation between cation and temperature induced changes in the physical-chemical properties of the zoospore plasma membrane, in vivo, observed with ESR spectroscopy, and the effects of temperature and ions on zoospore differentiation and viability (10,11). The ESR spectra previously obtained for spin labeled zoospores were also interpreted as indicating the presence of two co-existing spin label populations which were in dynamic equilibrium with each other. Of these two populations, it was the one characterized as the more rigid which seemed to determine the temperature limits of zoospore viability and be involved in the regulation of encystment.

Experiments with aqueous dispersions of the total lipids (phospho-, glyco-, and neutral lipids) extracted from zoospores indicated that of three "break points" observed *in vivo*, with ESR (T_L, T_M, and T_H), at least the lower (T_L) and upper (T_H) were due to bulk changes in the properties of the zoospore lipids.

The purpose of this report is to expand upon these earlier results, especially, 1.) to determine which lipid components give rise to the phase transformations observed in the total lipid extract preparations, 2.) to indicate some general properties concerning the interactions of these components with other lipids and ions, and 3.) to suggest how these properties may affect zoospore differentiation. Part of the results obtained in this study were presented previously (12).

MATERIALS AND METHODS

Organism, Medium and Growth

Cultures of *Blastocladiella emersonii* were routinely grown on PYG agar at 22°C as previously described (13). Zoospores were harvested from first generation plants with a 5 mM Morpholinopropane sulfonic acid (MOPS) + 10 mM $CaCl_2$ solution (pH 6.7). The resulting zoospore suspension was then collected, filtered to remove germlings and plants, and pelleted by centrifugation (2000 x g for 5 minutes at room temperature).

Lipid Isolation and Characterization

Lipids were immediately extracted from the zoospores, the proteins removed, and the separated lipids fractionated into neutral, glyco-, and phospholipid classes as previously described (14). Both lipid extraction and subsequent fractionations were carried out under N_2. All solvent evaporations were carried out at 40°C or less, and samples were stored under N_2 at -20°C. Solvents were spectra grade or redistilled.

Individual lipid components were separated by TLC on Silica gel G, Silica gel H, and Aflasil plates (Supelco) using chloroform/methanol/water (65:25:4 v/v/v) and petroleum ether/diethyl ether/acetic acid (80:20:1 v/v/v). The separated lipids were detected with iodine vapor, sulfuric acid charring, ninhydrin, Rhodamine 6 G, and $FeCl_3$ in H_2SO_4 (for sterols). Zoospore neutral lipids were separated on a preparative scale by TLC on silica gel G plates. Bands were found by blowing iodine vapor (iodine crystals in a Pasteur pipette) over end strips of the TLC plates. The four major spots were marked and the in-between silica gel bands scraped off. The lipids were extracted from the silica gel using chloroform/methanol (1:1 v/v) as solvent, and stored under N_2 at -20°C. Comparable experiments with lipids (dipalmitoyl phosphatidylcholine) indicated a recovery rate of 90 to 93%. Lipid components were identified

by co-chromatography with standards (Supelco), color reactions to the detection sprays, and comparison to published analyses of B. emersonii zoospore lipids (14).

Fatty Acid and Glycolipid Sugar Analysis

A Hewlett-Packard gas chromatograph, Model 402, equipped with a flame ionization detector was used. Peak areas were determined with a Hewlett-Packard integrator, Model 3380 A. Fatty acyl methyl esters were separated by GLC chromatography at 170°C on a 2m x 2mm glass column containing 3% SP-2330 on 80 - 100 mesh, Supelcoport. The fatty acyl methyl esters were identified by comparing their retention times to standard mixtures (Supelco). Glycolipid sugars were analyzed on a 3% OV-1 column at 190°C according to the method of Yang and Hakamori (15). This method allowed the detection of amino sugars. The sugars were identified by comparison of their retention times with those obtained for standards.

Preparation of Aqueous Dispersions and Spin-Labeling Procedure

The spin label 5-nitroxystearate was used for these ESR studies. Aqueous dispersions of the lipid components were made by mixing aliquonts of the desired lipids in chloroform/methanol (2:1 v/v) and drying the mixture first under N_2, then under vacuum. The sample was resuspended on a vortex stirrer into 5 mM MOPS buffer (pH 6.7)

with a glass bead. The ratio of buffer to lipid was adjusted to give a final lipid concentration of at least 5.0 mg/ml. The suspension was then sealed in a screw cap test tube under nitrogen and sonicated (125 Watt sonic water bath). An ethanolic solution of the spin-label was then added to the lipid dispersion, the tubes resealed and the dispersion sonicated for another 5 to 10 minutes to incorporate the label. The concentration of the spin-label was less than 0.2% (wt./wt.). Control experiments indicated that the small amounts of ethanol added did not alter the spectra measured. For some experiments ethylene glycol (final conc. 33%) was added to the sample just before transfer into the ESR cuvette to depress the freezing point. Previous experiments had shown that this procedure did not affect the temperature at which break points were found in plots of $2T_{||}$ vs. temperature (11). In experiments to test the effects of Ca^{2+} ions on membrane rigidity, $CaCl_2$ (final conc. 10 - 20 mM) was added both to samples which had just been tested without calcium, and to freshly prepared samples just before transfer into the ESR cuvette. The effect of calcium was found to be the same in both cases. Since the addition of both K^+ and Ca^{2+} ions could cause aggregation, ions were added after the formation of the dispersion immediately before transfer into the EPR cuvette. Control experiments indicated

that the aggregation itself did not affect rigidity.

Spin-Label Measurements and Analysis

ESR measurements were made with a Varian X-band spectrometer (Model E-112). The temperature was regulated with a Varian variable temperature controller and monitored inside the cuvette with a calibrated thermocouple connected to a digital readout meter (Omega model 250). All spectra were recorded at power and modulation amplitude settings which were previously determined to be below those causing saturation or line width broadening. The spectra were analyzed with a Varian 620/L-100 computer. The experimental plots of the hyperfine splitting parameter ($2T_{||}$), and the $H_A/(H_A + H_B)$ ratios vs. temperature, (where H_A and H_B are the heights of the peaks A and B of Figure 4, respectively), were analyzed in terms of linear components by fitting regression lines to appropriate sections using the method of least squares. The hyperfine splitting parameter $2T_{||}$ is indicative of motional properties of the spin probe in the membrane. A high value of $2T_{||}$ is associated with restricted motion or increased rigidity and a low $2T_{||}$ value corresponding to a decrease in rigidity. This empirical method of analysis has been frequently used in the interpretation of results obtained from ESR experiments, and the temperatures at which break points are observed (indicated by the intersections of straight

lines) are in good agreement with those obtained by other physical-chemical techniques (16,17,18).

RESULTS

Lipids - Analytical Studies

The percentages of neutral, glyco- and phospholipid fractions found in zoospores were 56, 12 and 31%, by weight. This is similar to the results previously reported for B. emersonii zoospores (14). The major phospholipids detected by TLC were phosphatidylcholine and phosphatidylethanolamine. These were previously shown to account for 55 and 22%, respectively, of the zoospore's phospholipids (14). A minor difference found was the absence in our preparations of lysophosphatidylcholine and phosphatidic acid (lysophosphatidylethanolamine not determined) which had been previously observed (14). Both lysophosphatidylcholine and phosphatidic acid could be detected by TLC if the samples were exposed to air or heated above 40°C for too long. The glycolipid fraction was composed of monoglycosyldiglycerides, diglycosyldiglycerides and a small amount of polyglycosyldiglycerides. These components have previously been shown to comprise 18, 70 and 12%, respectively, of the glycolipid fraction (14). The neutral lipid fraction was resolved into seven components with the solvent system employed. The four major components were

triglycerides, sterols, diglycerides and free fatty acids. These four components have previously been reported to account for 30, 13, 9 and 8%, respectively, of the total neutral lipid fraction (14).

The fatty acid composition of the total, phospho- and glycolipid fractions are shown in Table I. The major fatty acids detected were palmitic (16:0), oleic (18:1), γ-linolenic (18:3) and arachidonic (20:4). The phospholipid fraction was significantly enriched for palmitic and arachidonic acids in comparison with the glycolipid fraction. The glycolipid fraction in turn contained more oleic and γ-linolenic acids than the phospholipid fraction.

Analysis of the glycolipid sugars indicated that the major sugars present were glucose (83%), and mannose (15%). No N-acetylglucosamine was detected.

Lipids - ESR Spectroscopy

Phase transformations were previously observed with zoospores in vivo, and in the zoospore total lipid extract (10). In multicomponent systems, i.e., biomembranes, such phase transformations can be correlated to lipid phase transitions, lateral phase separations or lipid clusters. To ascertain whether these phase transformations preferentially involved either the zoospore phospholipids or glycolipids, aqueous dispersions of each lipid class were spin-labeled and

TABLE I

FATTY ACYL COMPOSITION[1] OF TOTAL LIPIDS, PHOSPHOLIPIDS, NEUTRAL LIPIDS AND GLYCOLIPIDS OF B. EMERSONII ZOOSPORES.

LIPID	FATTY ACYL COMPOSITION (MOLE %)[2]								UNSAT./SAT FATTY ACYL GROUPS (MOLE/MOLE)
	16:0[3]	16:1	18:0	18:1	18:2	γ-18:3	20:0	20:4	
TOTAL LIPID (4)	27.8	--[4]	2.6	17.9	5.2	16.5	1.1	28.9	2.2/1
PHOSPHOLIPID (5)	19.7	1.3	---	5.6	5.4	23.7	3.0	40.6	3.3/1
GLYCOLIPID (4)	12.0	1.0	---	14.6	6.2	34.2	8.3	23.4	3.8/1
NEUTRAL LIPID (4)	18.6	2.5	5.8	25.9	4.1	9.9	1.2	31.8	2.9/1

1. Determined as the methyl esters by GC analysis

2. Values are averages of multiple experiments; number of experiments indicated behind lipid name

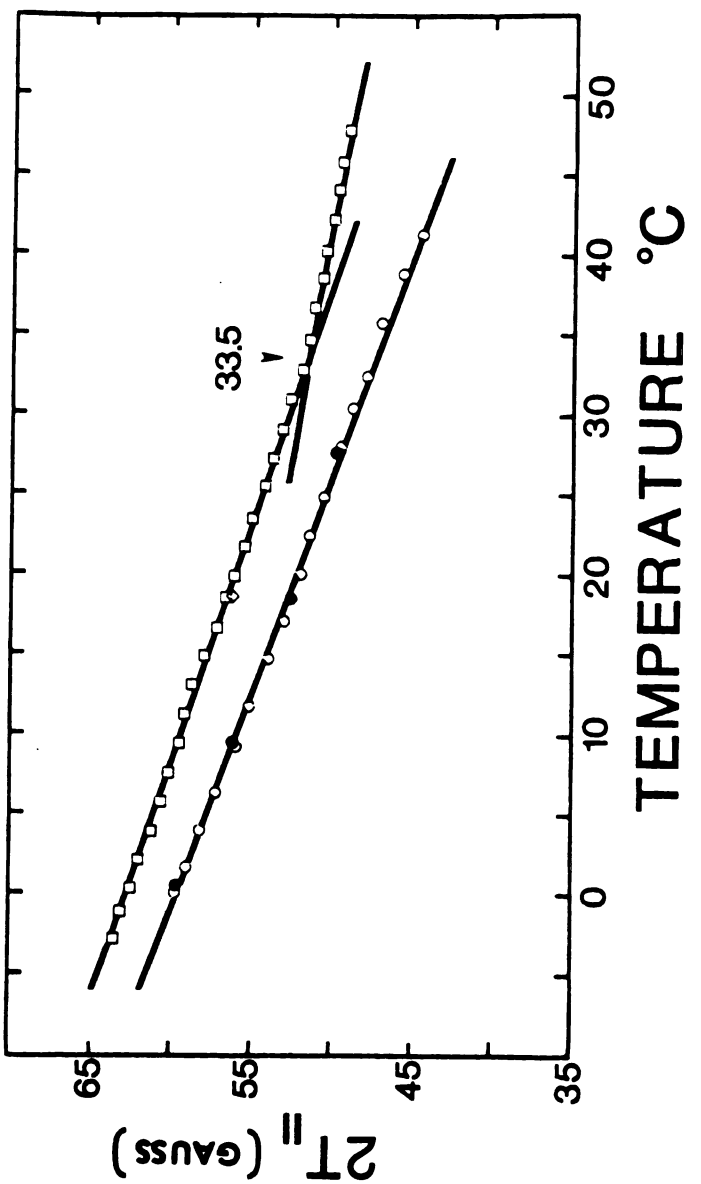
3. Number to the left of the colon refers to the number of carbon atoms; number to the right of the colon refers to the number of double bonds

4. Values less than 1%

analyzed as a function of temperature (Figure 1). A break point was observed in the glycolipid dispersions at 33 ± 1°C. A second break point was observed at 2 ± 1°C with glycolipid dispersions in the presence of ethylene glycol when measured over the temperature range -12°C to 20°C (data not shown). These break points are essentially the same as those found in plots of $2T_{||}$ vs. temperature for total lipid extract dispersions, i.e. 3 ± 1°C and 31 ± 1°C, and similar to the lower and upper break points (T_L and T_H) obtained in experiments with spin-labeled zoospores, <u>in vivo</u>, 5 ± 1°C and 33 ± 1°C respectively (11). No break points were found in the phospholipid samples over the temperature range tested.

Aqueous dispersions of zoospore phospholipids were more fluid than glycolipid dispersions over the entire temperature range as measured with the 5-NS probe. The absence of any break points and the greater relative fluidity of the phospholipid dispersions are consistent with the high percentage of polyunsaturated fatty acyl chains present (Table 1) and the phase transition temperatures of phosphatidylcholine liposomes containing unsaturated fatty acyl chains (19,20). This difference in membrane fluidity varied with temperature (Figure 1), the glycolipid dispersions being characterized by $2T_{||}$ values 3 gauss greater than the phospholipid dispersions at 0°C, and about 4 gauss greater at 22°C (growth

Figure 1 Plot of $2T_{||}$ vs. temperature for phospholipid and glycolipid dispersions labeled with 5-NS. Glycolipids, □ — □ ; phospholipids, ○ — ○ ; phospholipids + 20 mM $CaCl_2$, ● — ● .


2T∥ (Gauss)
65
55
45
35
0
10
20
30
40
50
TEMPERATURE °C
33.5

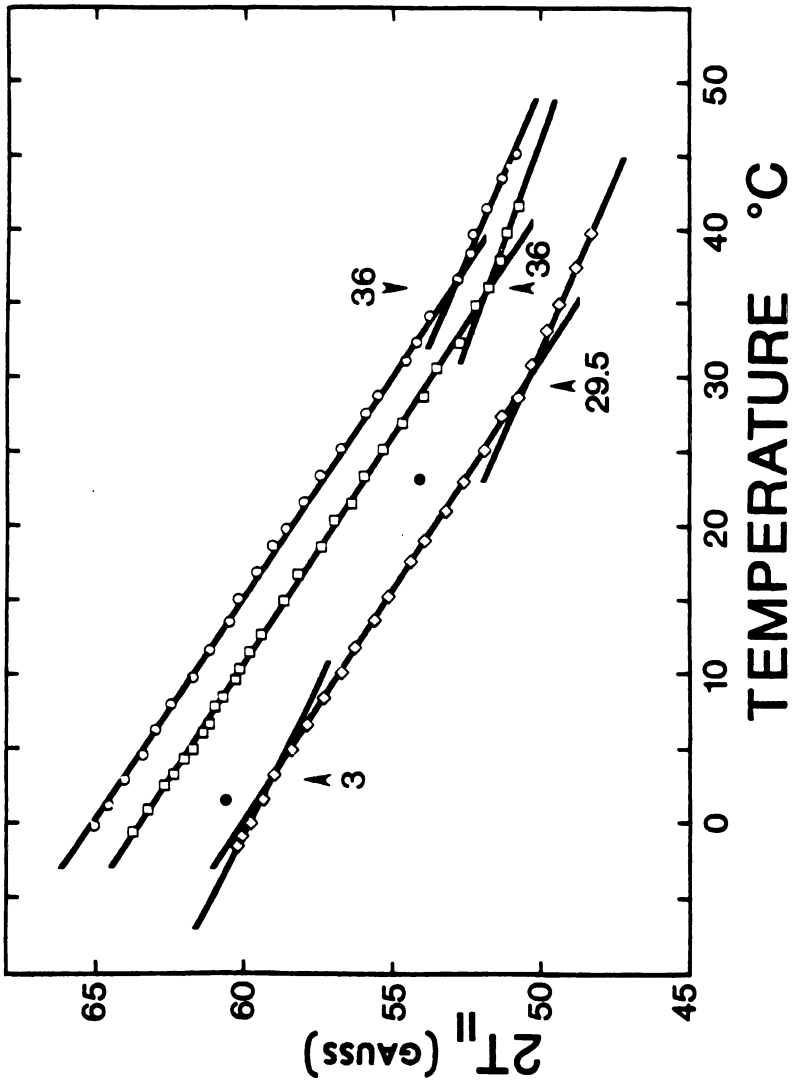
temperature). This difference increased above the 33 ± 1°C break point to 5 - 6 gauss.

The presence of 10 mM $CaCl_2$ did not change the $2T_{||}$ values obtained with the phospholipid dispersions (Figure 1). Glycolipid dispersions, in contrast, were affected by calcium ions, the membrane becoming 1.5 - 2.5 gauss more rigid at all temperatures (Table II, Figure 2).

The effects of zoospore phospholipid and cholesterol addition on the observed properties of the glycolipids were measured as a function of temperature using lipid mixtures (Figure 2). At a glycolipid to phospholipid ratio of 1 to 1 (wt./wt.) the upper break point (T_H) was observed at 29 ± 1°C, and the lower one (T_L) at 3 ± 1°C. In mixtures of glycolipid/phospholipid/cholesterol (1:1:1 wt./wt./wt.) the temperature range of the transition was significantly broadened, T_H occurring at 36 ± 1°C. The lower break point (T_L) was downshifted below the temperature range measured (below -2°C). The incorporation of cholesterol into the lipid mixture increased the rigidity of the dispersion as reflected by a 3 - 4 gauss increase in $2T_{||}$ over the entire temperature range.

In the presence of 10 mM $CaCl_2$ the $2T_{||}$ values obtained for both the glycolipid/phospholipid (1:1 wt./wt.) and glycolipid/phospholipid/cholesterol (1:1:1 wt./

Figure 2 Plot of $2T_{||}$ vs. temperature for mixed lipid dispersions labeled with 5-NS. Glycolipids/phospholipids (1:1 wt./wt.), ◇—◇; glycolipids/phospholipids (1:1 wt./wt.) + 10 mM $CaCl_2$, ●—●; glycolipids/phospholipids/cholesterol (1:1:1 wt./wt./wt.), □—□; glycolipids/phospholipids/cholesterol (1:1:1 wt./wt./wt.) + 10 mM $CaCl_2$, O——O. In neither case did the addition of Ca^{2+} ions change the temperature at which T_H occurred.


2T⊥ (gauss)
65
60
55
50
45
0
10
20
30
40
50
TEMPERATURE °C
36
36
29.5
3

wt./wt.) dispersions increased by 1 - 2 gauss. However, the addition of calcium did not change the temperature at which T_H occurred in either dispersion.

To further characterize the interactions possible between various lipid components, aqueous dispersions were made from specific lipids, spin-labeled, and the hyperfine splitting parameter ($2T_{||}$) measured at 23°C before and after $CaCl_2$ addition (Table II). Aqueous dispersions of the total lipid extract were found to have a very low $2T_{||}$ value, indicating a relatively fluid membrane. Addition of $CaCl_2$ increased the $2T_{||}$ value by 5 gauss. Both of these results seem to be due to the presence of triglycerides in the mixture, as indicated by the values obtained for the glycolipid/triglyceride dispersions and the phospholipid/neutral lipid and glycolipid/neutral lipid samples.

Addition of $CaCl_2$ to the more rigid glycolipid sample resulted in an increase in $2T_{||}$, whereas there was no affect on the more fluid phospholipid dispersion.

Aqueous dispersions of phospholipid/glycolipid mixtures at different lipid/lipid ratios had an intermediate $2T_{||}$ value. At a phospholipid/glycolipid ratio of 5 to 1 (wt./wt.), the addition of $CaCl_2$ did not increase the $2T_{||}$ value obtained. In contrast, at a 1 to 1 (wt./wt.) ratio, the presence of calcium ions increased the $2T_{||}$ value by 1.5 gauss, which is similar

TABLE II

EFFECT OF LIPID COMPOSITION AND CALCIUM IONS ON THE HYPERFINE SPLITTING PARAMETER ($2T_{||}$) OBTAINED FOR AQUEOUS DISPERSIONS LABELED WITH 5-NS AND MEASURED AT 23°C

Lipid Composition (by wt.)	$2T_{||}$ (gauss)[1]		
	$-Ca^{2+}$	$+Ca^{2+}$[3]	Δgauss
Total lipid extract	48.50	53.50	5.0
Phospholipids	50.50	50.50	0.0
Glycolipids	54.25	55.75	1.5
Phospholipid/Glycolipid (5:1)	52.50	52.50	0.0
Phospholipid/Glycolipid (1:1)	52.50	54.0	1.5
Phospholipid/Neutral lipid (1:1.5)	50.0	51.5	1.5
Glycolipid/Neutral lipid (1:3)	55.75	59.25	3.5
Glycolipid/Triglycerides[4]	48.25	53.25	5.0
Glycolipid/Free Fatty Acids[2,4]	55.25	57.25	2.0
Glycolipid/Sterols[4]	58.5	59.5	1.0
Glycolipid/Diglycerides[4]	55.25	56.75	1.5
Glycolipid/Phospholipid/Cholesterol (1:1:1)	56.0	57.5	1.5

1. Values are averages of three experiments. The standard deviations were ±0.25 gauss. Resolution of any single $2T_{||}$ value was ±0.1 gauss.
2. The free fatty acid region found on the TLC plates were actually composed ofthree components, only one of which was a free fatty acid. This was determined by direct methylation of the carboxylic acid using diazomethane, followed by TLC. The methyl esters of the fatty acids migrated differently than the free fatty acids, exposing two previously hidden components. The nature of these components is unknown, although they do contain fatty acyl chains.
3. Final concentration 10 mM $CaCl_2$
4. Component ratio same as existed in total lipid extracts

to that obtained for the pure glycolipid dispersions.

Mixtures of glycolipids and different neutral lipid fractions had variable $2T_{||}$ values, with the glycolipid/sterol dispersion having the highest $2T_{||}$ value of any sample tested, 58.5 gauss, and the glycolipid/triglyceride mixture the lowest, 48.25 gauss. Thus, there was more than a 10 gauss difference in the $2T_{||}$ values obtained for these two mixtures, indicating a significant effect of the different neutral lipids on the zoospore's glycolipids. The addition of $CaCl_2$ to these mixtures resulted in a similar increase in $2T_{||}$, 1 - 2 gauss. The difference in the $2T_{||}$ values (Table II) for glycolipid/neutral lipid (1:3) dispersions vs. those of the glycolipid dispersion probably are caused by the presence of sterols in the neutral lipids, since sterols markedly enhanced the $2T_{||}$ values obtained for the glycolipid/sterol dispersions.

The competitive effects of Ca^{2+} and K^{+} on zoospore glycolipid dispersions at 23°C are shown in Table III. The addition of $CaCl_2$ to the sample increased the $2T_{||}$ value obtained by about 2 gauss. This calcium effect could be reversed by EDTA. The addition of KCl did not reverse the calcium effect, nor could it inhibit it.

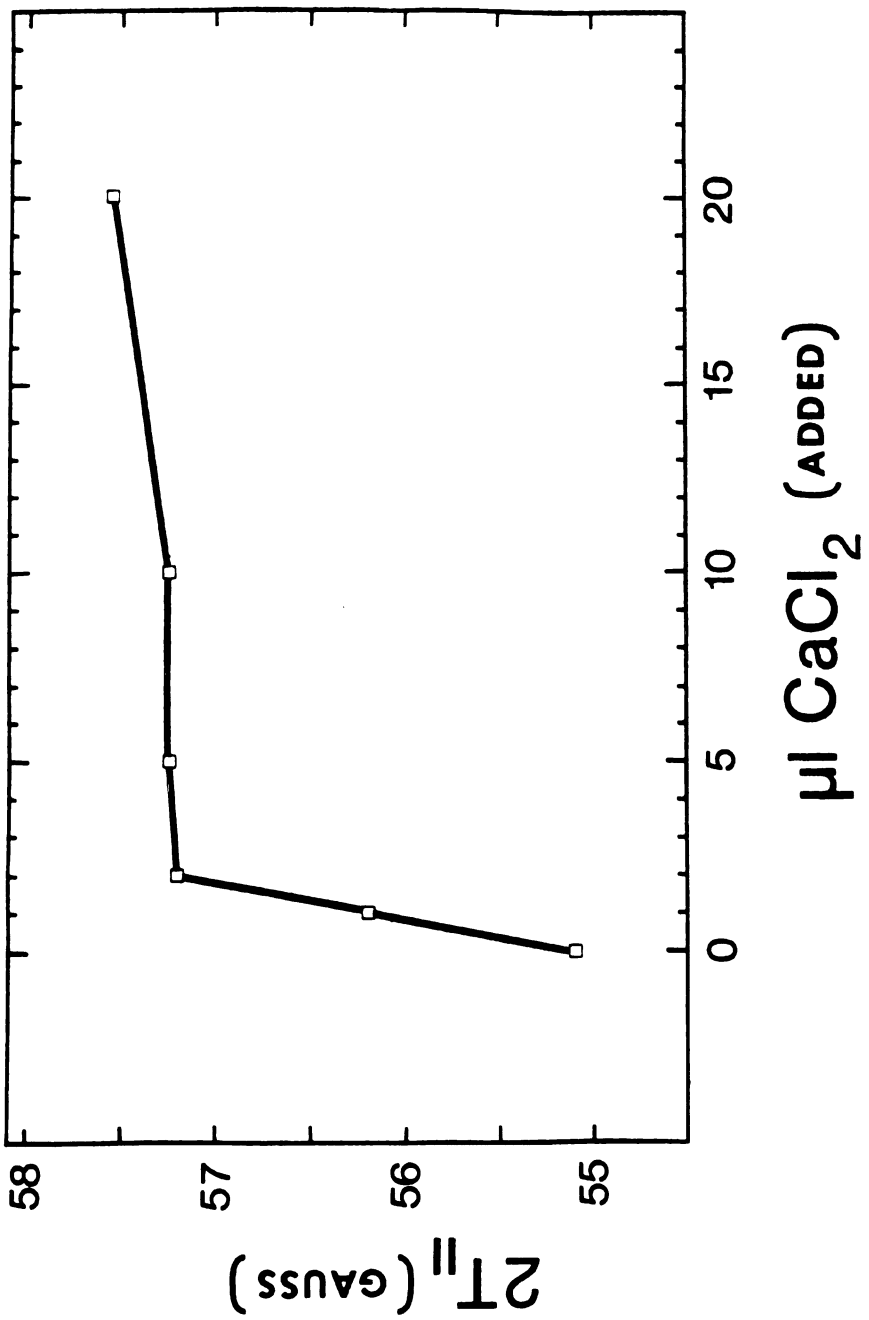
To approximate the stoichiometry of the glycolipid/calcium ion interaction, glycolipid dispersions were

TABLE III

EFFECT OF CALCIUM AND POTASSIUM ION ADDITION ON THE HYPERFINE SPLITTING PARAMETER ($2T_{||}$) VALUES OBTAINED FOR GLYCOLIPID DISPERSIONS LABELED WITH 5-NS, AND MEASURED AT 24°C (pH 6.7).

SEQUENCE	$2T_{||}$ (gauss)
I	
GLYCOLIPID ONLY	54.0
+ 20 mM $CaCl_2$	56.7
+ 100 mM KCl	56.4
+ 20 mM EDTA	53.9
II	
GLYCOLIPID ONLY	54.2
+ 50 mM KCl	54.5
+ 10 mM $CaCl_2$	56.4
+ 20 mM EDTA	53.9

Figure 3 Plot of $2T_{||}$ vs. total amount (µl) of $CaCl_2$ added to glycolipid dispersions labeled with 5-NS. Temperature was 23°C. Lipid concentration was 17 mg/ml. Volume was 0.5 ml. pH was 6.7. Millimolarity of $CaCl_2$ solution is 2 x µl added.


2T⊥ (Gauss)
58
57
56
55
0
5
10
15
20
μl CaCl2 (ADDED)

spin-labeled with 5-NS and the hyperfine splitting parameter ($2T_{||}$) measured as a function of increasing calcium ion concentration. The results of such an experiment are shown in Figure 3. Assuming 1.) a molecular weight for the glycolipids of 1000, which is close to the molecular weight of the major glycolipid component, the diglycosyldiglycerides, 2.) that the dispersion is composed of unilamellar vesicles with an outside/inside lipid ratio of 50/50 or 65/35, and 3.) that all of the calcium ions present are interacting with the outer lipid monolayer, one arrives at a glycolipid/calcium ion ratio of 2.1/1 for a 50/50 outside/inside lipid ratio and 2.7/1 for a 65/35 outside/inside lipid ratio.

ESR experiments with 5-NS labeled zoospores suggested the presence of two spin-label populations characterized by different degrees of spin-label immobilization (Figure 4) (11). The ratio (H_A/H_B) of these two spectral components, as measured by the peak height of the low field peaks, changed reversibly as a function of temperature. To determine if the glycolipids were involved in these changes, aqueous dispersions of the zoospore glycolipids were prepared, spin-labeled, and the spin-label populations analyzed as a function of temperature. Only one spin-label population was detected over the entire temperature range. This

Figure 4 Representative ESR spectra of 5-NS labeled zoospores recorded at different temperatures. The spectra are composed of two components, designated A and B.
_____ , 2°C; ....., 9°C;
------- , 36°C.

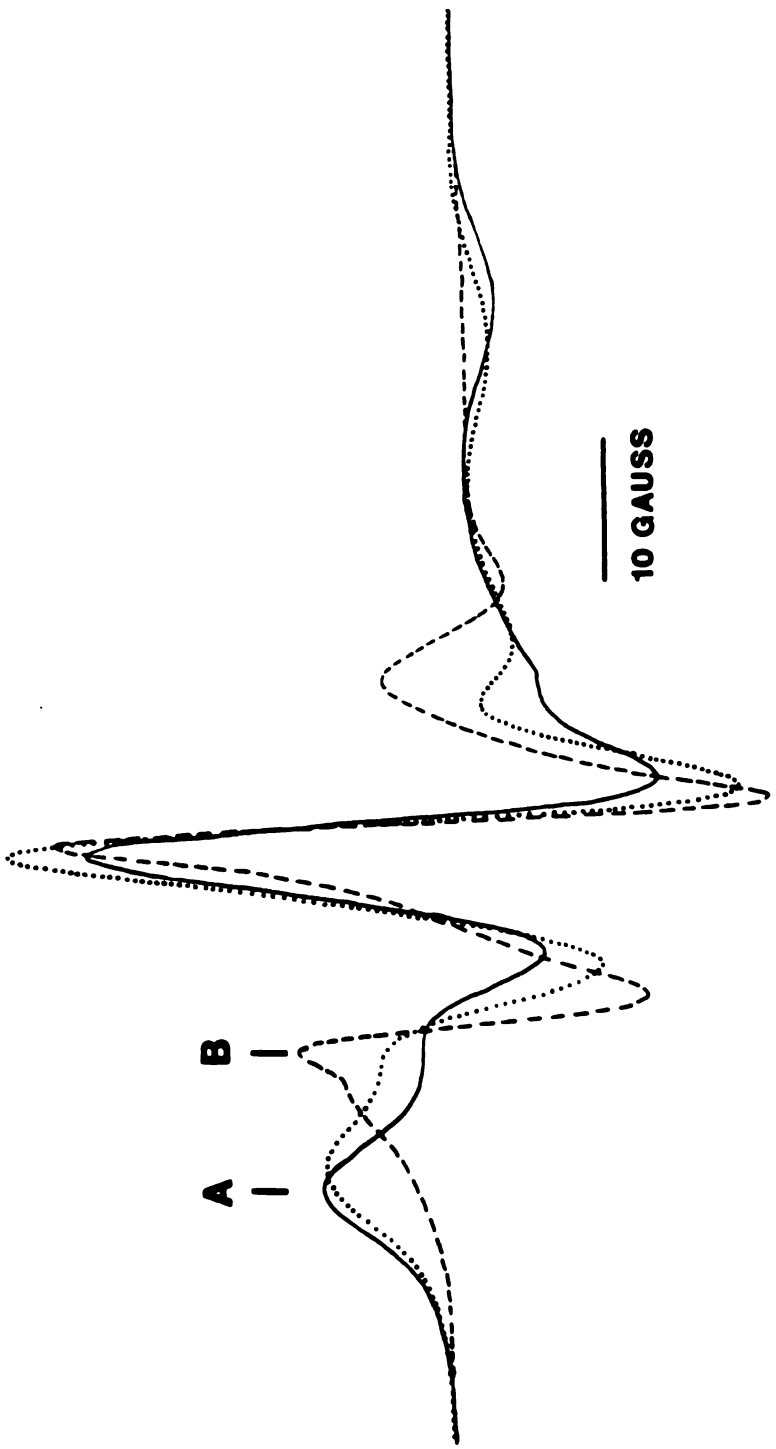
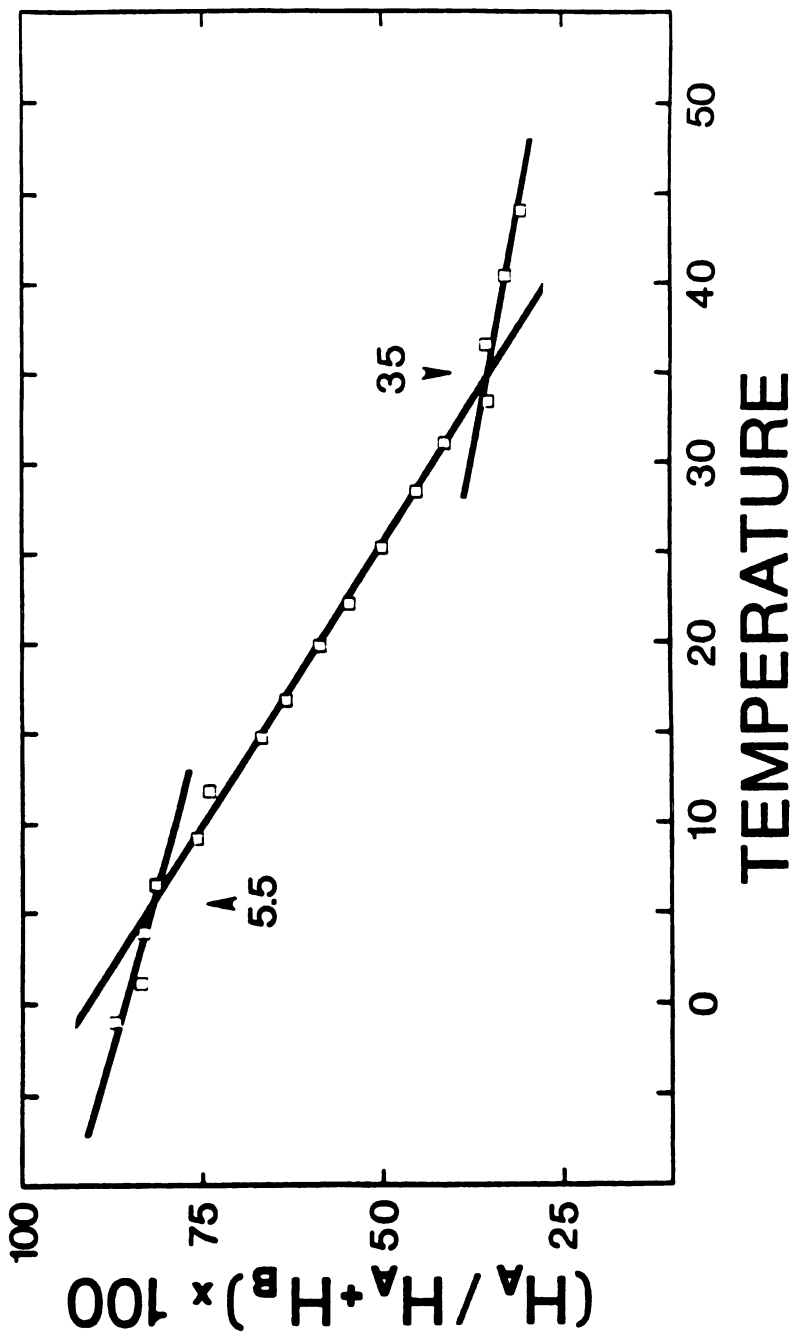

10 GAUSS
B
A

Figure 5 Plot of $\{H_A/(H_A + H_B)\}$ x 100 vs. temperature of glycolipid/neutral lipid dispersions (1:3 wt./wt.) labeled with 5-NS, where H_A and H_B correspond to the peak heights of the low field peaks.


100
75
50
25
$(H_A / H_A + H_B) \times 100$
0
10
20
30
40
50
TEMPERATURE
5.5
35

population corresponded to the more immobilized spectral component (A) observed in zoospore preparations. Since the possibility existed that the results obtained for zoospores *in vivo* required the presence of two different lipid classes, aqueous dispersions were made from a glycolipid/neutral lipid mixture (1:3 wt./wt.), spin-labeled and examined. The inclusion of the neutral lipids resulted in the presence of a more fluid component (B) into which the transfer of molecules from the more immobilized component (A) could be measured. When the ratio $\{H_A/(H_A + H_B)\}$ was plotted as a function of temperature, two break points were observed (Figure 5). The lower break point occurred at 5 ± 1°C and the upper break point was observed at 35 ± 1°C. Although dispersions of various lipid mixtures (Table II) were only measured at 23°C, the spectra obtained also suggest the presence of two populations in glycolipid/neutral mixtures and also in the total lipid extract.

DISCUSSION

The results indicate that it is the glycolipid fraction rather than the phospholipid fraction of the zoospore lipids which is mainly involved in the physical-chemical changes found in the zoospore total lipid extracts and in the zoospore plasma membrane, *in vivo* (11). This conclusion implies that the plasma

membrane of the zoospore contains a substantial amount of glycolipid. Indeed, chemical analysis of the isolated zoospore plasma membrane indicates that the major lipids present are diglucosyldiglycerides. Neutral lipids account for most of the remaining lipids, with the phospholipids comprising only a minor portion of the total (Leonards and Haug, unpublished).

The analytical studies on the glycolipids indicate that the major component of the glycolipid fraction is probably a diglucosyldiglyceride (Results and Ref. 12). Recent x-ray diffraction and NMR experiments have demonstrated that the gel-to-liquid-crystalline phase transition temperatures for mono- and diglucosyldiglycerides composed of mixed palmityl and oleoyl fatty acyl chains occurs between 15 and 25°C (21). This temperature range is similar to that observed for the zoospore glycolipid dispersions in our study. Together with the results obtained for zoospores *in vivo* (11), this correlation supports the hypothesis that the break points observed in the zoospore glycolipid dispersions represent the onset and completion of a gel-to-liquid-crystalline phase transition. The broadness of the transition observed in the zoospore glycolipid dispersions would therefore be due to the heterogeneity of the fatty acyl chains present (Table I).

The initial ESR studies with spin-labeled zoospores

were interpreted as indicating the presence of two spectral components (A and B) corresponding to two spin-label populations which seemed to co-exist in a dynamic equilibrium within the same membrane (Figure 4 and Ref. 11). This phenomenon was observed in lipid dispersions composed of glycolipids and neutral lipids, but was not present in glycolipid only dispersions. The results indicate that the more rigid spin-label population (component A) corresponds to the glycolipids and that the neutral lipids comprise the more fluid population (component B) present. The break points observed in the plots of $\{H_A/(H_A + H_B)\}$ vs. temperature (Figure 5) may therefore represent the movement of the glycolipid molecules from the more rigid population into the more fluid one as those molecules undergo a gel-to-liquid-crystalline phase transition. This conclusion is based on the assumption that the changes in spin-label populations observed as a consequence of increasing temperature reflect changes in the size of the two populations, and not only a specific redistribution of the 5-NS probe. Test experiments indicate that this is a valid assumption for this particular spin-label. The temperature at which these break points occurred, $5 \pm 1^{\circ}C$ and $35 \pm 1^{\circ}C$, are similar to the lower and upper (T_L and T_H) break points found with zoospores <u>in vivo</u> (11). This suggests that the viability limits of the

zoospore may be determined by the phase properties of the glycolipids.

The variability in the $2T_{||}$ values obtained for the different lipid mixtures indicates that the fluidity of the glycolipids was markedly influenced by the presence of other lipids. Both the increase in $2T_{||}$ observed for glycolipid/zoospore sterol dispersions, and the broadening of the temperature range of the phase transition in the presence of cholesterol, are similar to the results obtained for phospholipid/cholesterol mixtures (19,22,23). These effects are probably due to the inflexibility of the planar ring structure of the sterol and the steric restrictions placed on adjacent fatty acyl chains.

The $2T_{||}$ value obtained for the phospholipid/glycolipid dispersion mixtures was the average of the two, suggesting an extensive mixing of the two classes. Increasing the phospholipid to glycolipid ratio, however, did not further change the $2T_{||}$ value measured. Instead, the observable effect of $CaCl_2$ on the dispersion was eliminated. These results suggest that it is possible to dilute out the perturbations in the dispersion structure caused by the glycolipid/calcium interactions.

Calcium ions increased the rigidity of the glycolipid dispersions as measured by the 5-NS probe, but had no effect on the phospholipid dispersions (Figure 1,

Table II). These results are consistent with the analytic data (Results and Ref. 14) which demonstrated that the major phospholipids present were phosphatidylcholine and phosphatidylethanolamine, neither of which interact with Ca^{2+} ions at the concentrations used (24).

In contrast, glycolipids, especially diglucosyldiglycerides, have been shown to interact with Ca^{2+} ions (21). The nature of this interaction is not known. However, the fact that the inclusion of diglycerides, sterols or free fatty acids in the dispersion did not alter the effect of Ca^{2+} ions on the sample, even though the base $2T_{||}$ values were affected (Table II), suggests that Ca^{2+} ions interact with the lipid head-groups rather than the hydrocarbon core. In addition, the Ca^{2+} ion-induced increase in $2T_{||}$ observed could be eliminated by sufficiently increasing the phospholipid to glycolipid ratio (Table II). Since the major difference between the neutral lipids and phospholipids is the presence of the phospholipid head-group, this data is consistent with a Ca^{2+} ion/glycolipid head-group interaction.

The increase in $2T_{||}$ seen after Ca^{2+} ion addition was found over the entire temperature range, including both below and above T_L and T_H, but had no effect on the temperature at which break points were detected in plots of $2T_{||}$ vs. temperature (Figure 2 and unpublished). This result suggests that the effect

of Ca^{2+} ions on the glycolipids is relatively independent of the phase properties of the hydrocarbon core (gel or liquid-crystalline).

NMR experiments have indicated that the addition of Ca^{2+} ions results in an increase in the hydration capacity of the diglucosyldiglyceride head-groups (21). One explanation given for this increase was a conformational change in the head-group, presumably from an orientation relatively parallel to the plane of the bilayer to one where the sugar groups were more perpendicular to the surface, allowing the exposure of previously shielded hydroxyl groups. The results obtained in our experiments are consistent with this interpretation. A similar phenomenon has been reported for the effects of trivalent cations on lecithin head-group orientation (25).

The stoichiometry of the glycolipid/Ca^{2+} ion interaction was calculated to be 2.1 to 1 or 2.7 to 1, depending on the ratio of molecules in the inner and outer monolayers. These values are only an approximation, and should probably be considered as a lower limit since it is doubtful that all of the Ca^{2+} ions present in the solution were interacting with the glycolipid molecules. A glycolipid/Ca^{2+} calcium ion ratio of 4.4 to 1 has been previously reported to strongly affect the hydration capacity of a diglucosyldiglyceride (21).

Whether this ratio represents a minimum or maximum number, however, was not stated.

There was little change in the $2T_{||}$ values obtained for glycolipid dispersions in the presence of K^+ ions. In addition, the presence of K^+ ions had no effect on the Ca^{2+} ion-induced increase in $2T_{||}$ observed in the glycolipid dispersions (Table III). The inability of K^+ ions to either prevent or reverse the Ca^{2+} effect suggests that the two ions are not competing for the same interaction site. This result is important because in experiments with zoospores, the effect of Ca^{2+} ions on the physical-chemical properties of the zoospore plasma membrane could be reversed by the addition of K^+ ions (same ion concentrations) (11). Other studies have also demonstrated an immediate release of $^{45}Ca^{2+}$ from zoospores upon K^+ ion addition (26). Therefore, if the glycolipids were solely responsible for the Ca^{2+} ion effects observed <u>in vivo</u>, the reversal of these effects by K^+ ions must involve other membrane parameters, and not be due to the simple replacement of Ca^{2+} ions by K^+ ions.

In the initial studies with zoospores, three break points were observed (T_L, T_M and T_H) (11). The middle break point, T_M, has so far not been detected in any lipid dispersion. The absence of the middle break point suggests that either T_M is the result of

a lipid/protein interaction, or T_M is due to a lipid/lipid interaction which requires a specific organization of the membrane. These possibilities are presently being investigated.

Zoospore glycolipids play significant roles in the physiology of this organism. Besides the correlation between the break points obtained for the glycolipid dispersions and the temperature limits of zoospore viability previously observed, there is strong evidence which indicates that zoospore glycolipids are intimately involved in chitin biosynthesis, perhaps acting as the initial acceptor, or primer, for the polymerization of GlcNac units (27). These findings suggest that the physical-chemical properties of the zoospore glycolipids may also be involved in the regulation of chitin biosynthesis in this organism.

Finally, the observation that the zoospore plasma membrane contains substantial amounts of glycolipids, and very little phospholipid (no PA or PS detected) indicates that any general model for membrane fusion proposed must also be applicable to glycolipid membranes.

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SECTION III

ISOLATION AND CHARACTERIZATION OF THE PLASMA MEMBRANE OF *BLASTOCLADIELLA EMERSONII* ZOOSPORES

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Running title: Isolation of *B. emersonii* plasma membrane

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Abstract

A procedure for the isolation of the plasma membranes from zoospores of Blastocladiella emersonii, which involves the rupturing of the plasma membrane, is described. The ultrastructural organization of the zoospore permits the removal of the major cytoplasmic organelles as a single unit. Analysis of the membrane lipids indicated that the membrane is composed of glycolipids and neutral lipids, rather than phospholipids. The glycolipid fraction contained only diglycosyldiglycerides, and ≈90% of the glycolipid head-groups were glucose, indicating that diglucosyldiglycerides comprise the largest single group of lipids in the plasma membrane preparations (≈47% of total lipid by wt.). The membrane preparation was also characterized by the absence of triglycerides, although it did contain sterols. The preponderance of glycolipids in the plasma membrane preparations can account for the physical-chemical changes previously observed in zoospore plasma membranes in vivo, and in isolated plasma membrane preparations. The presence of glycolipids in both the plasma membrane and γ-particles, which directly interact with each other, via vesicle fusion, to form the chitinous cell wall, and the biochemical role of glycolipids in chitin synthesis in this organism, suggest that the glycolipids may be involved in the regulation of zoospore encystment.

Introduction

The role(s) of the plasma membrane in development and differentiation is presently an area of intense interest in biology. To date, most of the existing body of knowledge on plasma membranes has been derived either from studies on prokaryotes, erythrocytes, and model membranes, where the difficulties associated with eukaryotic systems are avoided, or from studies on Saccharomyces cerevisiae (Durán et al, 1975; Santos et al, 1978) and Neurospora crassa (Scarborough, 1975), where a cell wall must first be removed, either enzymatically or by the use of cell surface mutants. A desirable alternative would be to find an organism which 1) exhibits the differentiation and development characteristic of an eukaryotic organism, and 2) is structurally organized so as to minimize possible contamination from cytoplasmic organelles, and potential artifacts related to cell wall removal.

Zoospores of the Chytridiomycete Blastocladiella emersonii, Cantino & Hyatt, fulfill both of these criteria, and are an excellent model system for studying the role(s) of the plasma membrane in cellular differentiation. Ultrastructurally, the zoospore is characterized by the absence of both a cell wall and an endoplasmic reticulum. In addition the nucleus, nuclear cap (ribosomes), and side-body complex, consisting of a mitochondrion, a single microbody, lipid globules, and

a single rootlet, are held together as a single internal complex of organelles by a backing membrane/nuclear membrane system. The only major cytoplasmic organelles not associated with this complex are the γ-particles.

Physiologically, the plasma membrane is intimately involved in the encystment process. The first detectable changes which occur during encystment involve the depolarization of the plasma membrane by cations (Jen & Haug, 1980; Brunt & Harold, 1980). These changes are followed by the induction of cell adhesiveness (Cantino et al, 1968), the irregular folding of the plasma membrane (Truesdell & Cantino, 1971), alterations in the cell surface as monitored by FITC-Concanavalin A (Jen & Haug, 1979), and the fusion of vesicles derived from the γ-particles with the plasma membrane (Myers & Cantino, 1974; Truesdell & Cantino, 1970, 1971). In addition, the encystment process does not seem to require either protein or RNA synthesis (Soll & Sonneborn, 1971; Lovett, 1975), suggesting that at least the initial stages of encystment involve alterations of already existing components. At the molecular level we have previously demonstrated a correlation between cation and temperature induced changes in the physical-chemical properties of the zoospore plasma membrane, in vivo, and the effects of temperature and cations on zoospore differentiation and viability (Leonards & Haug, 1980a).

A more detailed examination of the physical-chemical properties of the zoospore cell surface would be greatly facilitated by an isolated plasma membrane preparation. In this article we describe a simple method for the isolation of the zoospore plasma membrane, which is suitable for physiological and physical-chemical studies, and also characterize the membrane components.

Materials and Methods

Organism, Medium and Growth

Cultures of B. emersonii were routinely grown on PYG agar at 22°C as previously described (Cantino & Hyatt, 1953).

Isolation of the Zoospore Plasma Membrane

Petri plates containing first generation plants were flooded with a 5 mM Morpholinopropane sulfonic acid (MOPS) solution containing 10 mM $CaCl_2$ (pH 6.7) (Suberkropp & Cantino, 1972), when 10 to 20% of the zoospores has been released. The Petri plates were then left standing at room temperature for at least 2 h, usually 2.5 - 3 h. Under these conditions approximately 80% of the zoosporangia had discharged their zoospores, and they had been swimming for at least 1 h.

The zoospore suspension was then collected by filtration through Whatman #1 paper, which removed germlings and thalli, and pelleted by centrifugation

(1000 g, for 5 min) at room temperature. Cells were counted with a Petroff-Hauser chamber using aliquonts of the filtered zoospore suspension before centrifugation. After centrifugation the supernatant was decanted, residual supernatant being removed with a Pasteur pipette.

The cell pellet was gently resuspended in buffer and transferred into 12 ml centrifuge tubes. Additional buffer was then added to bring the final volume-to-pellet ratio to 10 to 1. The suspension was left standing for 1 h at room temperature to rupture the zoospores' plasma membranes, after which the tubes were centrifuged at 100 g for 10 min. to remove the membrane-bound nuclear cap/symphyomicrobody-lipid complexes and unbroken cells. The supernatant was removed with a Pasteur pipette, and transferred into another centrifuge tube. The cell pellet was then resuspended in buffer and re-centrifuged. This process was repeated a total of three times.

The supernatants from each centrifugation were also re-centrifuged at 100 g for 10 min. After re-centrifugation the supernatants were bulked and diluted with buffer (final volume 40 ml per 1-2 x 10^{10} cells harvested). The bulked supernatant was then filtered under mild suction through a 5 µm Millipore filter using two Whatman # 3 paper disks as pre-filters.

The filtrate was centrifuged at 30,000 g for 4

h at 4°C. After centrifugation the supernatant was removed by pipette. The pellet consisted of two layers. The bottom layer was clear and gelatinous. The plasma membranes formed the second layer, which was a white sheet of membrane material loosely overlaying the clear layer. The top layer was easily separated by even minimal agitation and removed with a Pasteur pipette. This top layer constituted the plasma membrane preparation used throughout the remainder of this study. (The entire procedure for the isolation of the plasma membranes is outlined in Figure 1.)

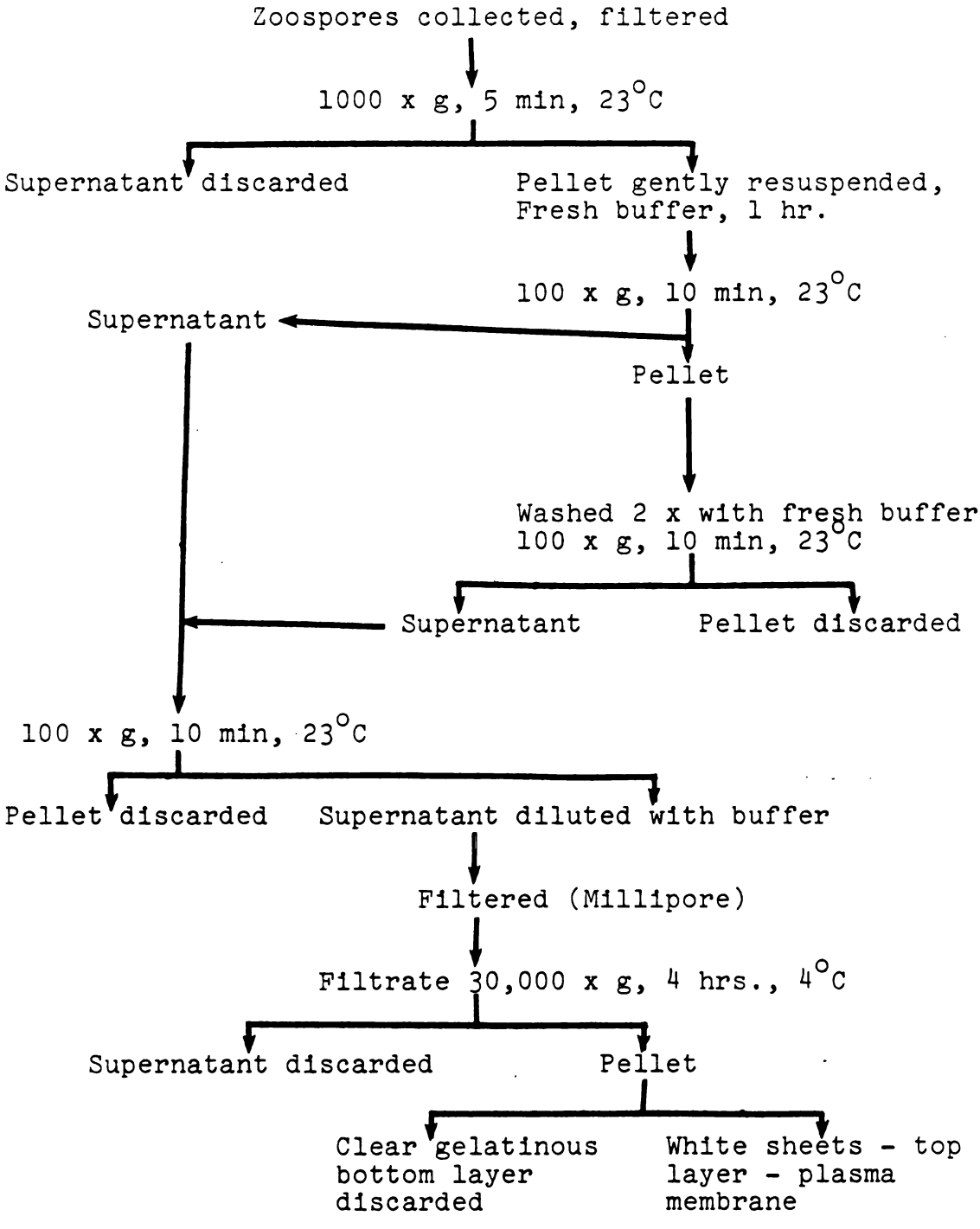
Enzyme Assays

Enzyme activities were assayed spectrophotometrically at 23°C. Succinic dehydrogenase activity (E.C. 1.3.99.1) was measured according to Hiatt (1961), to determine mitochondrial contamination. Isocitrate lyase activity (E.C. 4.1.3.1) was assayed according to Dixon and Kornberg (1959), to determine microbody contamination. Both methods have been used previously in studies on B. emersonii zoospores (Mills & Cantino, 1975).

Analytical Determinations

Protein was measured according to the modified Lowry method of Wang and Smith (1975) for membrane proteins. RNA was determined with the orcinol procedure (Schneider, 1957), using yeast RNA as reference, according to Myers and Cantino (1974). Zoospore γ-particles

Figure 1. Flow diagram of the procedure for isolating plasma membranes from zoospores of *B. emersonii*.


Zoospores collected, filtered
1000 x g, 5 min, 23°C
Supernatant discarded
Pellet gently resuspended, Fresh buffer, 1 hr.
100 x g, 10 min, 23°C
Supernatant
Pellet
Washed 2 x with fresh buffer 100 x g, 10 min, 23°C
Supernatant
Pellet discarded
100 x g, 10 min, 23°C
Pellet discarded
Supernatant diluted with buffer
Filtered (Millipore)
Filtrate 30,000 x g, 4 hrs., 4°C
Supernatant discarded
Pellet
Clear gelatinous bottom layer discarded
White sheets - top layer - plasma membrane

were tracked with neutral red (Myers and Cantino, 1974). The protein to lipid ratio was determined by combining three isolated membrane preparations, measuring the amount of protein/ml as above, isolating the membrane lipids, and weighing the lipid after drying under N_2 and vacuum.

Gel Electrophoresis

Membrane proteins were separated by gel electrophoresis using the discontinuous SDS buffer system of Laemmli (1970). The separating gels (10 cm) were 10% acrylamide, and the stacking gels (1 cm) were 5% acrylamide. The final SDS concentration of both the gels and the electrophoresis buffer was 0.1%. The membrane samples were prepared according to the method of Laemmli (1970), except that they were boiled for 3 minutes. The final protein concentration was 1-2 mg/ml. Protein standards (Boehringer Mannheim Gmblt. Biochemica) were co-electrophoresed in both separate tubes and in mixtures containing the membrane samples. The samples were electrophoresed at 5 mA/tube. Proteins were stained with Coomassie brilliant blue (Weber and Osborn, 1969). The stained gels were scanned spectrophotometrically at 550 nm, using a Gilford linear transport system, Model 2520.

Electron Microscopy

Isolated zoospore plasma membrane preparations were fixed for two hours with 5% glutaraldehyde in 0.1 M

phosphate buffer (pH 6.8), washed twice with buffer alone, and post-fixed overnight with 1% osmium tetroxide in buffer. The samples were then washed, dehydrated, and embedded in Epon-Araldite (Mollenhausers #1). Thin-sectioning was done with a Porter-Blum MT-2 Ultramicrotome. Silver sections were stained in saturated uranyl acetate, followed by saturated lead citrate, and viewed with a Phillips 300 electron microscope.

Radioisotope Studies

To quantitate the lipids present in the zoospore plasma membrane preparations, a zoospore suspension in liquid PYG medium containing 20 μCi/100 mls of (U-^{14}C) sodium acetate (New England Nuclear) at a final concentration of 5×10^{-4} M was used as the inoculation solution. The radioactivity of the lipid components, separated by TLC, was measured using a Packard Radiochromatogram Scanner (Model 7200). The various lipid components were quantitated by weighing paper cut-outs of the strip chart recordings.

Lipid Isolation and Characterization

Lipids were extracted from the plasma membrane preparations with chloroform/methanol (2:1, v/v) overnight under N_2. The sample was dried under N_2 at $\leq 40^{\circ}C$ and redissolved in Folsch Lower Phase. Non-liqid contaminants were removed by chromatography on G-25 Sephadex

according to the method of Radin (1969). The lipid sample was then dried under N_2 at $\leq 40^{\circ}C$, redissolved in chloroform/methanol (2:1, v/v), and stored at $-20^{\circ}C$ under N_2. As a control procedure, the modified lipid extraction method of Bligh and Dyer (Kates, 1972) was also used. Both techniques gave the same results.

The separation of the lipids into their phospho-, glyco- and neutral lipid fractions was carried out on silicic acid columns according to the method of Kates (1972).

Individual lipid components were separated on Silica gel G, Silica gel H, and Aflasil plates (Supelco) using chloroform/methanol/water (65:25:4, v/v), petroleum ether/diethyl ether/acetic acid (80:20:1, v/v), and diisobutyl ketone/acetic acid/water (40:25:5, v/v) as solvents. All solvents were either redistilled or spectra grade distilled in glass (Burdick and Jackson Laboratories, Inc.). The separated lipids were detected with iodine vapor, sulfuric acid charring, ninhydrin, Rhodamine 6G, and $FeCl_3$ in H_2SO_4 (for sterols) (Kates, 1972).

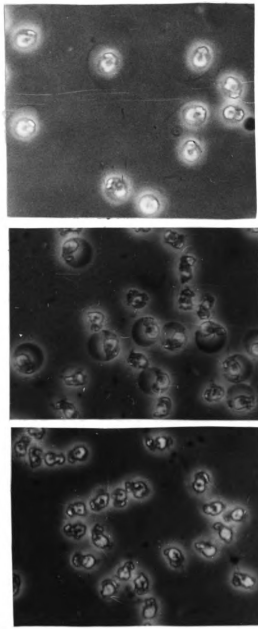
Lipid components were identified on the basis of 1) co-chromatography with purchased standards (Supelco), 2) co-chromatography with both extracted zoospore total lipids, and separated phospho-, glyco-, and neutral lipid fractions obtained from zoospores,

and 3) comparisons to previously obtained results for both total lipids extracted from zoospores (Mills & Cantino, 1974; Leonards & Haug, unpublished) and total lipids obtained from zoospore γ-particles (Mills & Cantino, 1978).

Fatty Acid and Isolated Lipid Sugar Analysis

A Hewlett-Packard Gas chromatograph, Model 402, equipped with a flame ionization detector was used. Peak areas were determined with a Hewlett-Packard integrator, Model 3380 A. Fatty acid methyl esters of the saponifiable total plasma membrane lipid fraction were separated by GLC chromatography at 170° on a 2 m x 2 mm glass column containing 3% SP-2330 on 80-100 mesh, Supelcoport. The fatty acid methyl esters were identified by comparing their retention times with those of standard mixtures (Supelco) and by comparison with the results obtained for isolated zoospore phospholipid and glycolipid fractions (Leonards & Haug, 1980b). Lipid sugars were analyzed on a 3% OV-1 column (Supelco) 2 m x 2 mm glass, at 190°C according to the method of Yang and Hakamori (1971). This method allowed the detection of amino sugars. The sugars were identified by comparisons of their retention times with those obtained for standards carried through the same procedure.

Figure 2. Phase-contrast micrographs of B. emersonii zoospores demonstrating the rupturing of the plasma membranes during the isolation procedure, x 660. A) Intact zoospores before the rupturing procedure. Note the appearance and organization of the cytoplasmic organelles, especially the nuclear cap (large bright crescent) and lipid globules associated with the side body (small bright bodies). B) Population of zoospores during the rupturing process. The plasma membrane has expanded, forming a large sphere within which the complex of cytoplasmic organelles can be observed. In some cases the cells plasma membrane has already ruptured, as judged by the loss of refractivity. C) The plasma membranes have ruptured, leaving the membrane bound nuclear cap/symphyomicrobody-lipid complexes intact. Note that both the nuclear cap and lipid globules are still bright and associated with the cytoplasmic organelle complexes.


A
B
C

Results

Isolation of the Zoospores' Plasma Membrane

Under the conditions employed the zoospores were ruptured, with the membrane-bound complex consisting of the nucleus, nuclear cap, mitochondrion, microbody, and lipid globules remaining as one unit. This process is illustrated in Figure 2 a, b, and c. (For a detailed description of the zoospore's internal organization see Myers & Cantino, 1974, Cantino & Mills, 1979). Note the appearance of the nuclear cap (large bright crescent and lipid globules (small bright bodies), which remain associated with each other. If the cells were exposed to too great an osmotic stress this membrane bound complex of cytoplasmic organelles also ruptured, and the lipid globules (small bright bodies) were dissociated from the other organelles. The appearance and relative locations of the nuclear cap and lipid bodies therefore provided an easy method for monitoring the integrity of this membrane bound complex of cytoplasmic organelles.

When the zoospores were resuspended in fresh buffer the plasma membranes expanded, forming large spheres within which the membrane bound complexes of cytoplasmic organelles could be observed (Figure 2b). Eventually the plasma membranes ruptured, as judged by the loss of refractivity in the phase microscope, forming small vesicles dispersed throughout the suspension.

Microscopic examination of the plasma membrane preparation indicated the presence of many vesicles 1-3 μm in diameter. The complexes of cytoplasmic organelles however were still intact, as indicated by the presence and relative locations of both the bright nuclear caps and bright lipid bodies (Figure 2c). After one hour more than 90% of the zoospores had been ruptured in this fashion.

The zoospores γ-particles were stained with neutral red, so that they could be observed during the isolation procedure (Myers & Cantino, 1974). After the centrifugation step usedto pellet the nuclear cap, symphyomicrobody-lipid complexes and unbroken cells, a dark-red pellet was found at the very bottom of the centrifuge tube. Microscopic examination revealed that under the conditions used to rupture the plasma membranes the γ-particles formed large red clumps which could not be easily broken up. Thus most, if not all, of the γ-particles were also removed by this centrifugation step. Any remaining γ-particles were found (as determined by their red color) at the bottom of the bottom layer after centrifugation at 30,000 g.

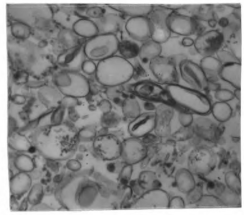
The flagella were removed from the suspension during the Millipore filtration step. Microscopic examination ascertained that they accumulated between the pre-filters and the Millipore filter.

The plasma membrane preparations were also

examined with electron microscopy (Figure 3). Vesicles, both single and multilayered, were observed. Some of the vesicles contained small particles (chemical analysis confirmed that they were not ribosomes), tentatively identified as "Blastocladiella polysaccharide" (Myers & Cantino, 1974). These particles are normally found throughout the cytoplasm, and were probably trapped in the vesicles when the plasma membranes ruptured.

Although microscopic examination of the zoospores during the plasma membrane isolation procedure indicated that contamination by other organelles was very small, chemical and enzymatic assays were also performed. Since plasma membranes have not been isolated from any Chytridiomycete, there are, as yet, no positive enzymatic markers to definitively distinguish the zoospore plasma membrane. However, it was possible to assay for a number of possible contaminants because of research on the isolation of various cytoplasmic organelles from B. emersonii (Lovett, 1963; Myers & Cantino, 1974; Mills & Cantino, 1975). Succinic dehydrogenase activity was assayed to determine mitochondrial contamination. Isocitrate lyase activity was measured to measure microbody contamination. Neither enzyme was detected in the plasma membrane preparations, although both enzymes were found in the pellet containing unbroken cells and the membrane bound nuclear cap/symphyomicrobody-lipid complexes.

Figure 3. Electron micrographs of the isolated plasma membrane preparation from zoospores of B. emersonii. Both unilamellar and multilamellar vesicles are present. Small particles were observed apparently within some of the vesicles. These particles may be "Blastocladiella polysaccharide", which are present throughout the cytoplasm of the zoospore. A) x 25,000 B) x 52,000



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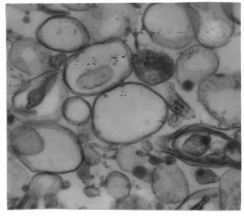

B

RNA was assayed to measure contamination by the nuclear cap ribosomes and the γ-particles. No RNA was detected in the plasma membrane preparations (less than 1 μg/ml based upon limits of standards), 99.6 to 99.9% of the RNA (relative to whole cells) being recovered in the pellet containing the membrane bound nuclear cap/symphyomicrobody-lipid complexes and unbroken cells.

Characterization of the Plasma Membrane Preparation

The plasma membrane preparations were composed of approximately 51% protein, and 49% lipid (by weight). These values are based upon the average value obtained by combining three membrane preparations and measuring the protein present with the modified Lowry procedure. The lipids were measured by extracting them from the combined membrane preparations, separating them from non-lipid contaminants on a Sephadex column, drying, and then weighing (wt. lipid for three combined preps. 2.95 mg).

The individual lipid components present in the plasma membrane preparations were quantitated by measuring the radioactivity of the ^{14}C labeled lipids. The major lipid components present in the plasma membrane preparations were glycolipids and neutral lipids, the percentages of glyco-,phospho-, and neutral lipids being 47.0, 11.35, and 41.65, respectively (Table I, Figure 4a). Sterols and diglycerides accounted for 14.0% of the

TABLE I

Comparative quantities of lipid classes in plasma membrane preparations

Lipid Component	Expt. #1	Expt. #2	Ave.
Phospholipid	12.3[1]	10.4	11.35
Glycolipid	45.2	48.8	47.00
Neutral lipid	43.5	39.8	41.65

[1]Values are percentages of total radioactivity determined from the total surface area of individual peaks from scans.

Figure 4. Representative labeling patterns on thin-layer chromatograms for the total lipid extracted from isolated plasma membrane preparations. Lipids were labeled with ^{14}C. A) Sample chromatographed with Chl/MeOH/H_2O (65:25:4 v/v) as solvent. Peak one corresponded to the phospholipids present, peak 2 to the glycolipids and peak 3 to the neutral lipids. B) Sample chromatographed with pet. ether/ diethyl ether/acetic acid (80:20:1 v/v) as solvent. Peak one corresponds to glycolipids, phospholipids and some neutral lipids. Peak 2 corresponds to diglycerides and sterols and peak 3 to the free fatty acids and two unknown compounds. Arrowhead marks the origin.

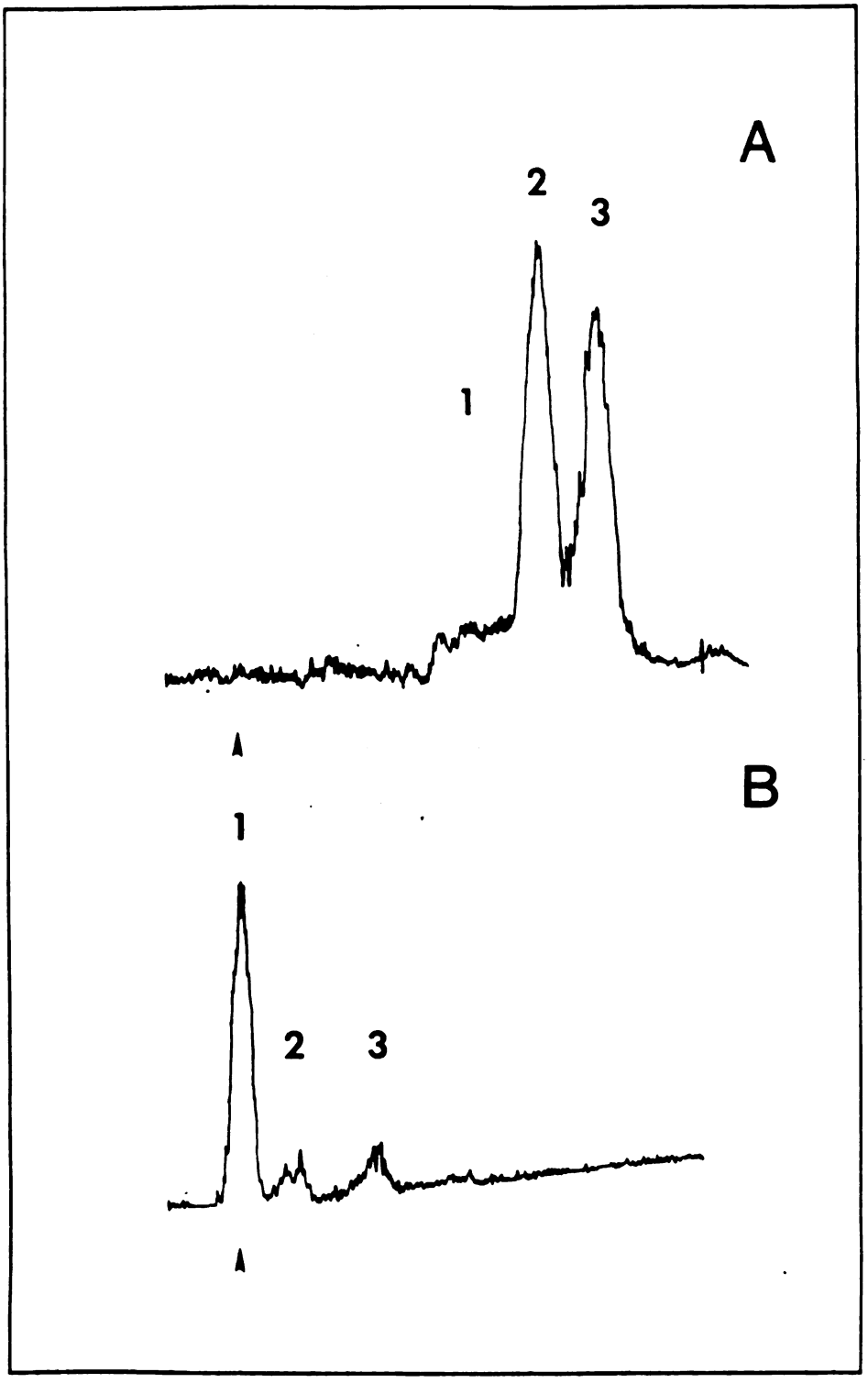
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Table II

Comparative quantities of lipid components in plasma membrane preparations

Lipid Components	Expt.#1	Expt.#2	Expt.#3	Ave.
Peak #1 (PL + GL + NL)	79.8[1]	75.0	63.7	72.8
Peak #2 (sterols + diglycerides)	9.0	14.9	18.4	14.1
Peak #3 (f.f. acids + two unknown compounds)	11.2	9.6	17.9	12.9

[1]Values are percentages of total radioactivity determined from the total surface area of individual peaks from scans.

radioactivity of the membrane preparation lipids. It should be noted however that sterols contain approximately one half of the number of carbon atoms found in both phospholipids and glycolipids. The actual number of sterol molecules will therefore be greater than the percentage of total radioactivity measured. (If this peak was due to sterols only, the value would thus be 28%). Free fatty acids and other co-migrating components comprised another 13% ofthe total (Table II, Figure 4b).

The lipids extracted from the plasma membrane preparations were chromatographed on Silica gel G plates, with petroleum ether/diethyl ether/acetic acid (80:20:1, v/v) as solvent, to further separate and identify the neutral lipids present. Six major components were detected, all of which were also present although in seemingly different relative concentrations, in the whole cell lipid extracts (Figure 5). The plasma membrane preparations were characterized, however, by the distinct absence of triglycerides, as determined by radioactive labeling (Figure 4b), iodine vapor, and sulfuric acid charring (Figure 5).

To further characterize the lipids present in the plasma membrane preparations, a few microliters of a highly concentrated lipid sample, were chromatographed simultaneously with the remainder of the sample (Figure 6), which has been separated into its phospho-, glyco-,

Figure 5. Thin-layer chromatogram of the total lipid extracted from isolated zoospore plasma membranes (B) and the total lipid extracted from whole cells (for comparison) (A) demonstrating the absence of triglycerides in the plasma membrane preparation. Solvent was pet. ether/diethyl ether/acetic acid (80:20:1 v/v). O, origin; 1 and 2, unknowns; 3, diglycerides; 4, sterols; 5, unknown; 6, free fatty acids + two unknown components; 7, triglycerides. Plates were charred with sulfuric acid.

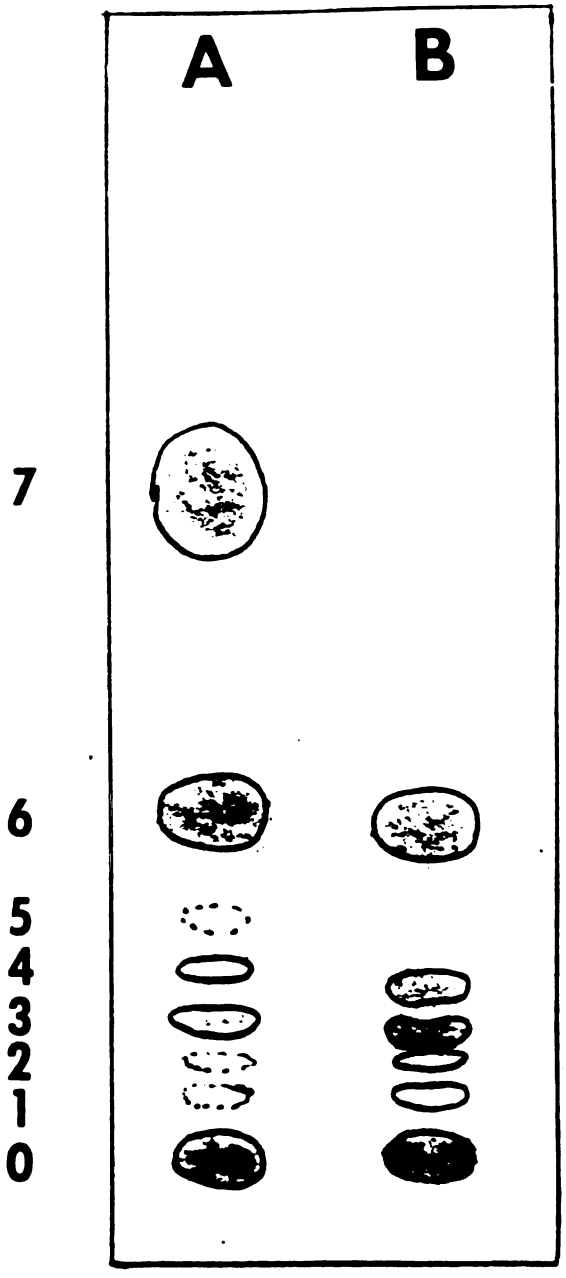
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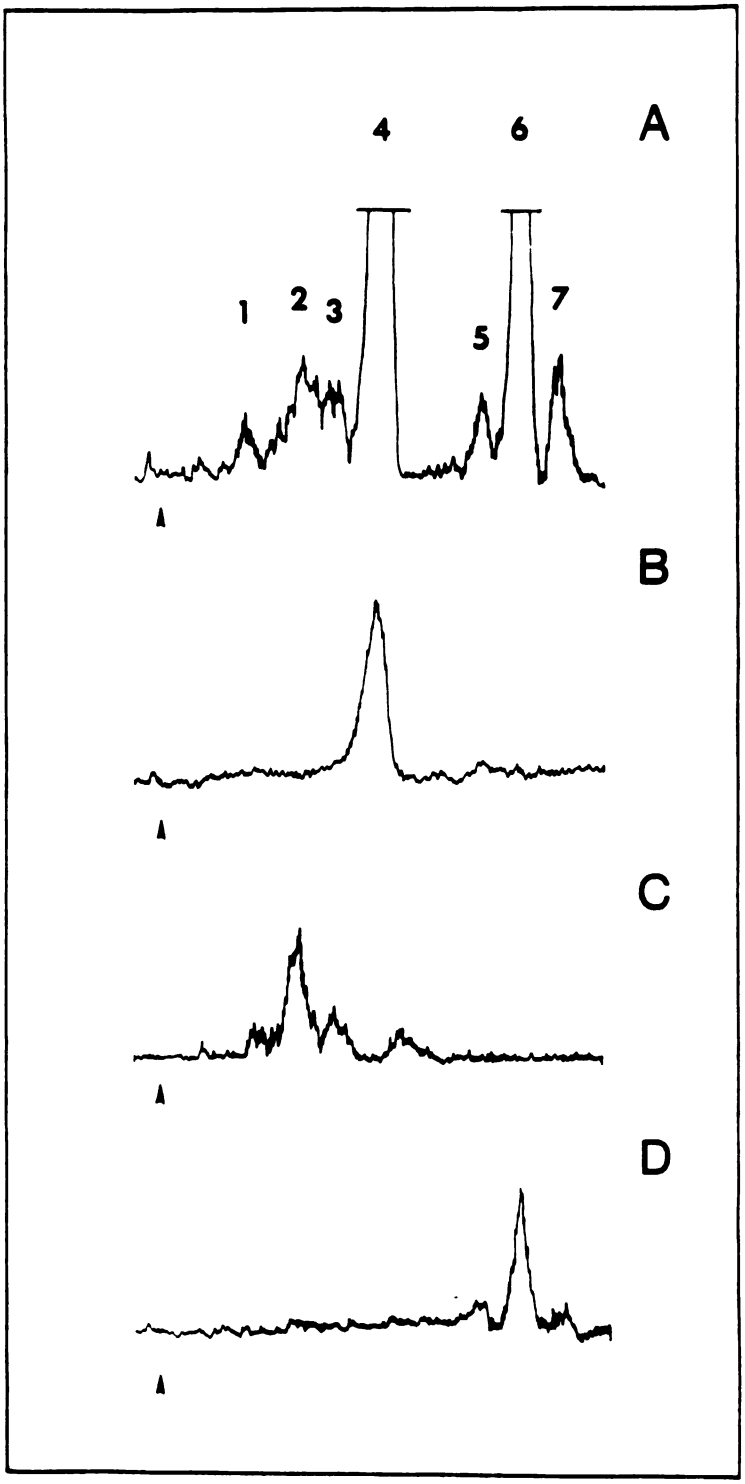
Figure 6. Representative labeling patterns on thin-layer chromatograms for the lipids extracted from the isolated plasma membrane preparations of B. emersonii zoospores. Lipids were labeled with ^{14}C. Samples were chromatographed with Chl/MeOH/H_2O (65:25:4 v/v) as solvent. A) The total lipid fraction. Seven lipid components were detected, the two main components (peaks 4 and 6) were allowed to go off-scale to permit detection of the minor components. The samples were also separated into phospho-,glyco- and neutral lipid classes before thin-layer chromatography to further qualitatively identify the components. B) Glycolipid fraction, containing only diglycosyldiglycerides. C) Phospholipid fraction, containing predominantly phosphatidylcholine. C) Neutral lipid fraction. Arrowhead indicates the origin.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and neutral lipid fractions by silicic acid chromatography. Seven lipid components were detected in the plasma membrane preparation lipid extracts. The two main components, peaks 4 and 6, were allowed to go off-scale to permit detection of the minor components (Figure 6a). The glycolipid fraction (Figure 6b) was composed entirely of diglycosyldiglycerides, corresponding to peak 4, and thus was the main lipid component present in the plasma membrane preparations (ave. 47% of total). The phospholipid fraction (Figure 6c) contained four components, the major one being phosphatidylcholine (7-8% of total). Peak 3 (2-3% of total) seemed to contain more than one component, phosphatidylcholine being identified in addition to phosphatidylinositol. Peaks 1 and 4 (1-2% of total, each) were identified as lysophosphatidylcholine (degradation product) and phosphatidylethanolamine. Peaks 5, 6 and 7 were found in the neutral lipid fraction (Figure 6d), but could not be individually identified in this solvent system.

The fatty acyl compositions of the total cell lipids and the lipids isolated from the plasma membrane preparations are shown in Table III. The major fatty acids detected were palmitic (16:0), arachidonic (20:4), and γ-linolenic (18:3). The membrane preparation lipids were enriched in plamitic and arachidonic acids in comparison with the total cell lipid extract, but

Table III

Fatty acyl composition[1] of total and plasma membrane lipids extracted from B. emersonii zoospores

Lipid	Fatty Acyl Composition (Mole %)								Unsat./Sat. Fatty Acyl Groups (Mole/Mole)
	16:0[3]	16:1	18:0	18:1	18:2	γ-18:3	20:0	20:4	
Total Lipid (4)[2]	27.8	---[4]	2.6	17.9	5.2	16.5	1.1	28.9	2.1/1
Plasma Membrane (3)	34.6	---	3.0	6.8	5.3	15.5	1.9	32.9	1.6/1

[1]Determined as the methyl esters by GC analysis

[2]Values in parentheses indicate number of separate experiments

[3]Numbers to the left of the colon refer to the number of carbon atoms; number to the right of the colon refers to the number of double bonds

[4]Values less than 1%

Table IV

Comparative composition of glycolipid sugars from plasma membrane preparations and whole cells[1,2]

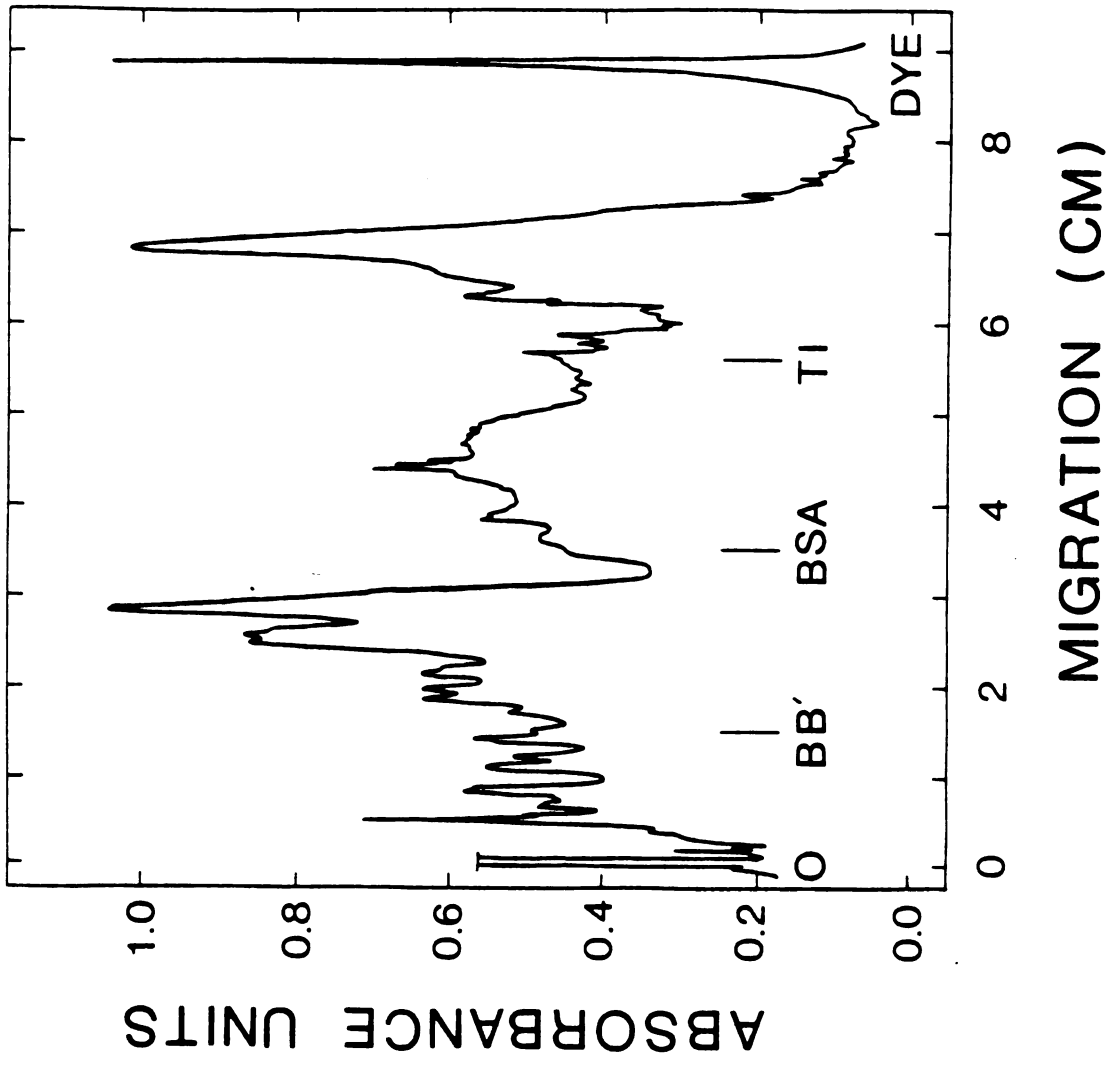
Glycolipid Sugar	Plasma Membrane Preparations (Mole%)			Whole Spores (Mole%)
	Expt.#1	Expt.#2	Ave.	
Glucose	90.17	91.33	90.75	82.47 ± 1.56[3]
Mannose	4.82	5.24	5.03	14.04 ± 2.37

[1]Only glucose and mannose were quantitated, three other peaks were detected, but were less than 2%.

[2]Determined by GLC analysis

[3]Average of three experiments ± S. D.

Figure 7. SDS-gel electrophoresis recording of absorbance vs. migration distance (cm) of plasma membrane proteins stained with Coomassie brilliant blue. Corresponding position of molecular weight standards: BB', and subunits of RNA-polymerase from E. coli (average 160,000 daltons); BSA, bovine serum albumin (68,000 daltons); and TI, trypsin inhibitor (21,500 daltons). O,origin.


ABSORBANCE UNITS
1.0
0.8
0.6
0.4
0.2
0.0
O
BB'
BSA
TI
DYE
0
2
4
6
8
MIGRATION (CM)

contained significantly less oleic acid (18:1). The plasma membrane preparation lipids also had a larger percentage of saturated fatty acids than the total cell lipid extract.

Analysis of the glycolipid sugars indicated that the major sugars present in the plasma membrane preparation lipids were glucose (≈90%) and mannose (≈5%) (Table IV). The glycolipid fraction separated from the total cell lipid extracts in comparison contained glucose (≈83%) and mannose (≈15%). No acetylglucosamine was detected in either glycolipid fraction.

SDS gel electrophoresis of the plasma membrane preparations demonstrated the presence of over 30 protein bands, as detected with Coomassie brilliant blue (Figure 7). Three major protein bands (peaks) were observed at 105,000, 90,000 and 13,000 daltons. Although PAS positive bands (possible glycoproteins) were detected, extensive interference presumably due to "B. emersonii polysaccharides" in the preparation made it impossible to determine which of the proteins detected may be a glycoprotein.

Discussion

The data presented in this paper indicate that it is possible to isolate the plasma membranes of B. emersonii zoospores by rupturing the cells. The procedure works because of the absence of a cell wall

wall and an endoplasmic reticulum, and the structural organization of the cytoplasmic organelles within the zoospore which permits the removal of the membrane bound nuclear cap/symphyomicrobody-lipid complex as a single unit. The rupturing process can be easily monitored microscopically. The removal of the cytoplasmic organelles, including the γ-particles, is also supported by the results of chemical and enzymatic assays of the plasma membrane preparations.

Although contamination from the above cytoplasmic organelles and organelle complexes was not detected, there is the possibility of contamination by a different source, the undifferentiated portion of the backing membrane proper. It has been reported (Truesdell & Cantino, 1971) that the undifferentiated portion of the backing membrane may be disrupted by improper fixation for electron microscopy, and that this portion of the backing membrane may become fractured even in ungerminating spores a few hours after sporulation. This possibility would not have been detected by microscopic examination during the isolation procedure, since the side body matrix, lipid bodies, and mitochondrion would still maintain their positions relative to one another (the differentiated portion of the backing membrane still being intact), and the nuclear cap/symphyomicrobody-lipid complex would still pellet as a single unit. There are at present, no

markers, structural, biochemical or physical (density), for this membrane fraction and thus no way of ascertaining its presence or absence in a preparation of any zoospore component. However, the undifferentiated portion of the backing membrane constitutes only a small fraction of the total backing membrane, and the surface area of the entire backing membrane, in turn, is only a fraction of that occupied by the plasma membrane. Thus, if the undifferentiated portion of the backing membrane is present, it is as a minor contaminant.

Analysis of the plasma membrane preparation lipids indicate that the zoospore plasma membrane is composed of glycolipids and neutral lipids (≈90%), rather than phospholipids. Moreover, the glycolipid fraction contains only diglycosyldiglycerides, and analysis of the glycolipid sugars indicates that approximately 90% of the glycolipid head-groups are glucose. These results indicate that diglucosyldiglycerides comprise the single largest group of lipids present in the zoospore plasma membrane (≈47% of total).

The preferential presence of glycolipids rather than phospholipids indicates that the zoospore plasma membrane has a glycolipid, specifically diglucosyldiglyceride based lipid matrix and not a phospholipid matrix. One important ramification of this finding is that any general mechanism proposed to explain the molecular basis

of membrane fusion, and cation effects on fusion, must be applicable to both types of lipid matrices. The significance of this point is emphasized by reports which indicate that in addition to the plasma membrane another cytoplasmic organelle, the γ-particle, also contains a substantial percentage of glycolipids (Mills & Cantino, 1978). It should be noted that these are the two cellular components which directly interact with each other, via vesicle fusion, to form the chitinous cell wall.

A comparison of the results obtained for the plasma membrane preparations with those reported for the zoospore γ-particle preparations (Mills & Cantino, 1978; Cantino & Mills, 1979) however also indicates marked differences in both their protein and lipid compositions. In the plasma membrane preparations more than 30 protein bands were detected by SDS-gel electrophoresis, as compared to seven protein bands for the γ-particle preparations (Cantino & Mills, 1979). The three major protein bands found in the plasma membrane preparations have molecular weights of 105,000; 90,000 and 13,000. No protein bands of equivalent molecular weights were reported as occurring in γ-particle preparations (Cantino & Mills, 1979). Instead SDS-gel electrophoresis of the γ-particle preparations indicated the presence of two major protein bands, with molecular weights of

40,100 and 37,425 which accounted for about 70% of the protein present.

With the significant exception of the substantial percentages of glycolipids present, the lipid composition of the plasma membrane preparations is also markedly different from that of the γ-particle preparations (Mills & Cantino, 1978). The only glycolipids present in the plasma membrane preparations are diglycosyldiglycerides. Gamma particles, in contrast, contain mono- and poly- glycosyldiglycerides in addition to diglycosyldiglycerides. Neutral lipids constitute almost 42% of the plasma membrane preparation lipids, whereas the neutral lipid fraction of the γ-particle preparations accounts for only 12% of the total lipids. The plasma membrane preparations were characterized, moreover, by the distinct absence of triglycerides. In contrast, triglycerides are the major component (≈60%) of the γ-particle preparations neutral lipid fraction. Finally, phospholipids comprise only a minor percentage of the plasma membrane preparations (≈11%) compared to their 37% value found in γ-particle preparations.

Electron spin resonance (ESR) spectroscopy studies of spin-labeled zoospores, in vivo, indicated that the effects of temperature and cations on the physical-chemical properties of the zoospore plasma membrane were closely correlated with the effects of temperature and cations

on zoospore differentiation and viability (Leonards & Haug, 1980a). Three break points were observed with ESR spectroscopy, indicating the presence of at least three distinct molecular changes within the plasma membrane. The lower (T_L) and upper (T_H) break points were independent of the external cation concentration, and closely correlated with the limits of zoospore viability. Experiments with aqueous dispersions of the isolated lipid components from zoospores indicated that these break points were the result of physical-chemical changes in the glycolipid rather than the phospholipid fraction (Leonards & Haug, 1980b). These experiments implied that the zoospore plasma membrane contained significant amounts of glycolipids. This implication is confirmed by the analysis of the membrane lipids.

In addition to the lower (T_L) and upper (T_H) break points, a third, middle break point (T_M) was observed with ESR spectroscopy of spin-labeled zoospores, in vivo, (Leonards & Haug, 1980a). This middle break point was markedly affected by the presence of Ca^{2+} ions, increasing from 11 ± 1°C (no $CaCl_2$ added) to 22 ± 1°C (10 mM $CaCl_2$). The temperature at which T_M occurred correlated very well with the effects of temperature and cations on zoospore encystment (Soll & Sonneborn, 1969; 1972; Truesdell & Cantino, 1971; Suberkropp & Cantino, 1972). Moreover Ca^{2+} ions were found

to interact strongly with the glycolipid fraction, but had no effect on the zoospore phospholipids (Leonards & Haug, 1980b). Calcium ions have been shown to change the hydration properties of diglucosyldiglycerides as a consequence of a cation/glycolipid interaction which may involve changes in the orientation of the glycolipid head-group (Wieslander et al, 1978). Given that glycolipids were found to be the major group of lipids present in the plasma membrane preparations these results suggest that a Ca^{2+} ion/glycolipid interaction may be involved in the molecular interactions associated with the middle break point (T_M) at the cell surface.

ESR experiments with spin-labeled aqueous dispersions of zoospore lipids have demonstrated that the physical-chemical properties of the glycolipid fraction can be modified by the presence of other zoospore lipids (Leonards & Haug, 1980b). Although zoospore glycolipid dispersions normally interact strongly with Ca^{2+} ions, the Ca^{2+} ion interaction is eliminated by phospholipids at phospholipid/glycolipid ratios of 5 to 1. Triglycerides modify the physical-chemical properties of aqueous dispersions of zoospore glycolipid even more dramatically, totally overwhelming the Ca^{2+} ion/glycolipid interaction. In contrast neither zoospore sterols nor other neutral lipid components had any effect on Ca^{2+} ion/glycolipid interactions, though they did alter the "fluidity" of

the dispersion. Given that cation interactions at the zoospore cell surface are intimately involved in the regulation of zoospore encystment, it is not unreasonable to expect that the presence of interfering components (phospholipids and triglycerides) would be minimized.

It has already been possible, using the isolated plasma membrane preparations, to ascertain that the middle break point (T_M), which was initially observed in spin-labeled zoospores, in vivo, and closely correlated with the effects of cations on zoospore encystment, is the result of a glycolipid/glycolipid or glycolipid/neutral lipid interaction rather than a lipid/protein interaction (Leonards & Haug, 1980c). These studies also indicated that the K^+ ion induced reversal of the Ca^{2+} ion effect required the presence of ATP, which seemed to be used to generate an "energized membrane state" (transmembrane potential). In related studies it has been possible to determine that K^+ ions trigger encystment by depolarizing the transmembrane potential of the zoospore (Jen & Haug, 1980). This K^+ ion effect has recently been also reported in intact zoospores by other researchers (Brunt & Harold, 1980).

The fact that zoospore encystment is a rapid process, requiring only a few minutes and no protein or RNA synthesis, combined with the above mentioned results, indicates that cation induced changes in the

physical-chemical properties of the plasma membrane are involved in the regulation of B. emersonii zoospore differentiation. The procedure described for the isolation of a plasma membrane preparation, which has already proven to be suitable for more detailed physiological and physical-chemical studies, should be very helpful in examining the molecular mechanisms involved in zoospore encystment.

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SECTION IV

POTASSIUM AND CALCIUM INDUCED ALTERATIONS IN THE LIPID INTERACTIONS OF ISOLATED PLASMA MEMBRANES FROM BLASTOCLADIELLA EMERSONII. EVIDENCE FOR AN ATP REQUIREMENT.[+]

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Running Title: PLASMA MEMBRANE Ca^{2+}, K^{+} INDUCED CHANGES

Footnotes:

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Abbreviations:

MOPS, Morpholinopropane sulfonic acid

EDTA, (Ethylenedinitrilo)tetraacetic acid

AMP-PNP, Adenylyl imidodiphosphate

5-NS or 5-nitroxystearate, {2-(3-Carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolindinyloxyl}

TLC, Thin-layer chromatography

Abstract

The physical-chemical properties of the isolated plasma membranes from zoospores of the Chytridiomycete Blastocladiella emersonii were investigated, with electron spin resonance (ESR) spectroscopy, using the spin label 5-nitroxystearate (5-NS). Both isolated plasma membranes and aqueous dispersions of the lipids extracted from the plasma membranes were spin-labeled and analyzed. Plots of the hyperfine splitting parameter ($2T_{||}$) vs. temperature indicated that the middle break point, T_M, initially observed in experiments with spin labeled zoospores in vivo {Leonards, K. S., & Haug, A. (1980a) Biochim. Biophys. Acta., in press} was the result of a lipid/lipid interaction (glycolipid/glycolipid or glycolipid/neutral lipid) rather than a lipid/protein interaction. This interaction was markedly affected by Ca^{2+} ions, which interacted directly with the lipid components, increasing T_M from 11 ± 1°C (Ca^{2+} removed by EDTA) to 21 ± 1°C (10 mM Ca^{2+}) in the lipid dispersions and 12 ± 1°C to 23 ± 1°C in the plasma membrane preparations.

The initial ESR studies on spin labeled zoospores in vivo had also demonstrated that the addition of K^+ ions could reverse the Ca^{2+} ion effect, downshifting T_M from 22 ± 1°C to 10 ± 1°C. The addition of K^+ ions to the isolated plasma membrane had no affect on T_M

indicating that K^+ ions do not simply replace Ca^{2+} ions, but exert their effect indirectly on the membrane. However, after the inclusion of ATP, K^+ ions could reverse the Ca^{2+} ion effect. It was determined that the ATP generated an "energized membrane" state which permitted the K^+ ions to reverse the Ca^{2+} effect. Since K^+ ions have been shown to depolarize the membrane potential in both zoospores and isolated zoospore plasma membrane preparations (generated by ATP), we suggest that the K^+ ion induced reversal of the Ca^{2+} ion effect, and therefore the change in the lipid/lipid interactions responsible for T_M, is a consequence of the K^+ ion induced depolarization of the membrane potential.

Introduction

Zoospores of the Chytridiomycete Blastocladiella emersonii have proven to be an excellent model system for studying the role(s) of the plasma membrane in eukaryotic cell differentiation and development. The plasma membrane is intimately involved in the differentiation process. Although zoospore encystment does not seem to require either protein or RNA synthesis (Soll & Sonneborn, 1971; Lovett, 1975), it does involve extensive membrane alterations. The first detectable changes which occur during encystment include the induction of cell adhesiveness (Cantino et al, 1968), alterations in cell surface monitored by FITC-Conconavalin A (Jen & Haug, 1979), and the fusion of vesicles derived from the γ-particles with the plasma membranes (Truesdell & Cantino, 1970; Myers & Cantino, 1974). In addition, the differentiation process is markedly affected by the zoospore's ionic environment, with Ca^{2+} ions inhibiting and K^{+} ions inducing encystment (Cantino et al, 1968; Soll & Sonneborn, 1969, 1972).

We have previously demonstrated a correlation between cation and temperature induced changes in the physical-chemical properties of the zoospore plasma membrane, in vivo, and the effects of temperature and cations on zoospore differentiation and viability (Leonards & Haug 1979a, 1980a). These studies indicated

the presence of three break points (T_L, T_M and T_H), as observed with ESR spectroscopy, in plots of $2T_{||}$, S, and f vs. temperature. Of the three, T_L and T_H were observed in zoospore total lipid extracts. Further studies on the isolated lipid components of the zoospore indicated that it was the zoospore glycolipids rather than the phospholipids which gave rise to T_L and T_H (Leonards & Haug, 1979b; 1980b).

However, it was the middle break point, T_M, which was affected by the external cation concentration. The cation-induced shifts in T_M were closely correlated with the temperature dependence and physiological effects of cations on zoospore differentiation, suggesting that the physical-chemical properties of the plasma membrane were involved in regulating the initial changes during zoospore encystment (Leonards & Haug, 1979a; 1980a).

The absence of the middle break point in both the total lipid extracts and in the zoospore phospholipid and glycolipid dispersions suggested that T_M is either the result of a lipid/protein interaction, or is due to a lipid/lipid interaction which requires a specific organization or lipid composition of the zoospore plasma membrane.

The purpose of this paper is to specifically study the middle break point, T_M, in isolated zoospore plasma membrane preparations in order to determine a.) is T_M

the result of a lipid/protein or lipid/lipid interaction, b.) are calcium ions involved in this interaction, c.) under what conditions can potassium ions reverse the calcium ion effect, as observed in zoospores *in vivo*, and d.) how do the properties of T_M observed in the isolated plasma membrane preparations compare to those obtained for zoospores *in vivo*?

Materials and Methods

Organism, Growth and Membrane Isolation

Cultures of Blastocladiella emersonii were routinely grown on PYG agar at 22°C as previously described (Cantino & Hyatt, 1953). Zoospores were harvested from first generation plants with a 5 mM Morpholinopropane sulfonic acid (MOPS) + 10 mM $CaCl_2$ solution (pH 6.7). The resulting zoospore suspension was then collected and filtered to remove germlings. Zoospore plasma membranes were isolated according to the method of Leonards and Haug (1980c). Because of the zoospore's ultrastructure it is possible to selectively rupture the cell osmotically, and remove the nuclear cap, nucleus, mitochondrion, and side body complex (microbody) as a single unit (there is no endoplasmic reticulum in the zoospore). The plasma membranes are then separated from these and the other cytoplasmic organelle, the γ-particles, by filtration and differential centrifugation.

The plasma membranes were resuspended in a minimal volume of supernatant (250-300 μl) which contained 5 mM MOPS + 10 mM $CaCl_2$ (pH 6.7). The supernatant was from the last step of the membrane isolation procedure, and was essentially a wash solution. Protein concentration varied between 3 and 4 mg/ml. These preparations were used immediately for the electron

spin resonance studies on isolated membranes.

Chemicals

All organic solvents were either redistilled or spectra grade distilled in glass (Burdick and Jackson Laboratories, Inc.). ATP (both disodium salt, Grade II, 95-98% and disodium salt from equine muscle, vanadium free, 99-100%), EDTA (tetrasodium salt, 99%), Morpholinopropane sulfonic acid (MOPS), Adenylyl Imidodiphosphate (AMP-PNP, 90-95%), and G-25 Sephadex were from Sigma Chemical Co. 5-nitroxystearate (5-NS) was from Syva Research Chemicals.

Lipid Isolation and Analytical Determinations

Plasma membrane lipids were extracted and isolated as previously described (Leonards & Haug, 1980c). Non-lipid contaminants were removed from the preparations by chromatography on G-25 Sephadex according to the method of Radin (1969). Protein removal was verified by protein determination before and after Sephadex chromatography. Protein was measured using the modified Lowry method of Wang and Smith (1975), with bovine serum albumin as the standard.

The lipid composition of the plasma membrane preparations was monitored by thin-layer chromatography (TLC) on Silica gel G and H using chloroform/methanol/water (65:25:4 v/v/v) and petroleum ether/diethyl ether/acetic acid (80:20:1 v/v/v) as solvents. Individual

lipid components were identified by co-chromatography with standards (Supelco), extracted zoospore total, phospho-, glyco-, and neutral lipid samples, and comparisons to previously obtained results (Mills & Cantino, 1974; Leonards & Haug, 1979b; 1980b).

Spin Labeling Procedure

Isolated membrane preparations were spin labeled as follows:

a.) the membrane preparation was sonicated in a bath sonicator for 10 minutes at room temperature to break up the membrane sheets and generate vesicles of a relatively uniform size,

b.) 5-nitroxystearate (5-NS) was added as an ethanolic solution (usually 1-2 µl) to give a final spin label/protein concentration $\leq$0.2% wt./wt.,

c.) the sample was again sonicated for 5 minutes to incorporate the spin label,

d.) sample was transferred into the ESR cuvette and spectra were obtained over the temperature range 0-30 ± 2°C,

e.) the sample was transferred back to the test tube, and

f.) KCl (final conc. 50 mM) and/or EDTA (final conc. 10 mM) were added, the

suspension mixed, and again transferred to the ESR cuvette for the next run.

This basic procedure was modified for specific experiments. In some cases, KCl and/or EDTA were added between steps c. and d. as a control. Alternatively, the sample was extracted with chloroform/methanol (2:1 v/v) at step e. for TLC. The effects of ATP, and AMP-PNP on T_M in isolated membrane preparations were measured using freshly prepared (kept on ice) solutions in 5 mM MOPS (pH 6.7) which were added to the sample immediately before step a.

Aqueous dispersions of the isolated plasma membrane lipids were made by drying the lipids first under N_2, and then under vacuum. The sample was resuspended on a vortex stirrer into 200-300 µl of 5 mM MOPS (pH 6.7) with a glass bead. The lipid concentration was between 3 and 5 mg/ml. The suspension was then sonicated in a sonic water bath, followed by steps b. through f. as above. Spin label concentration was 0.2% wt./wt. $CaCl_2$ (final conc. 10 mM) was added just before step d. ATP and/or KCl were added either just before step d., or at step f. EDTA was added at step f., or in control experiments just before step d.

Spin Label Measurements and Analysis

ESR measurements were made with a Varian X-band spectrometer (Moedl E-112). The temperature was

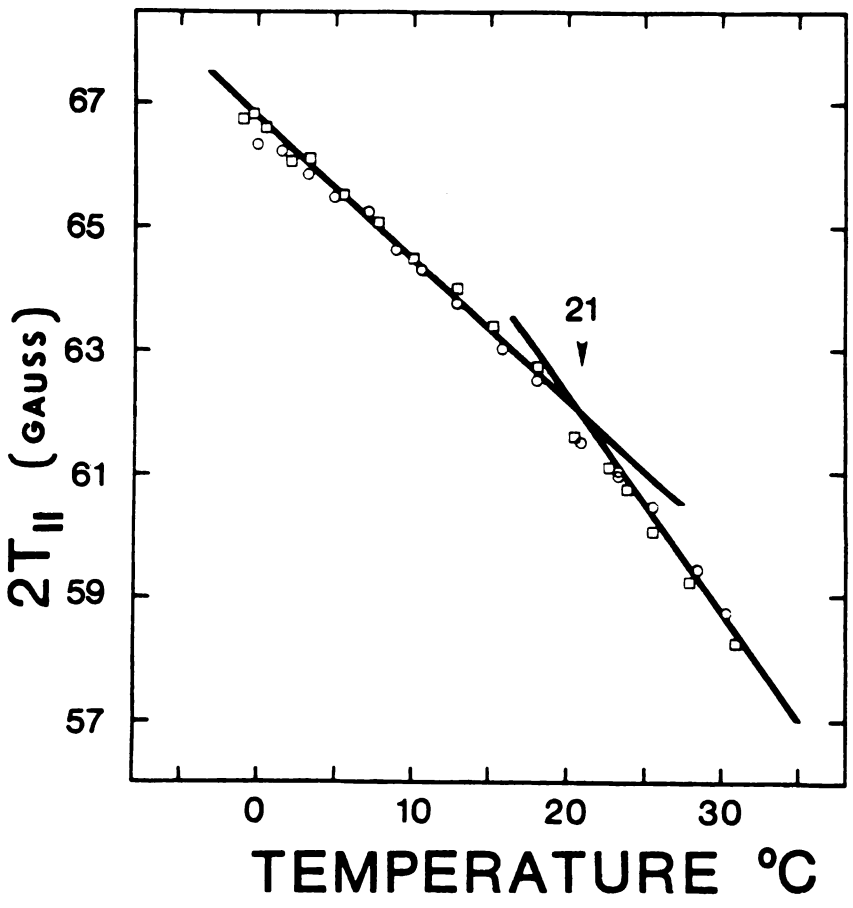
regulated with a Varian variable temperature controller and monitored inside the cuvette with a calibrated thermocouple connected to a digital readout meter (Omega model 250). All spectra were recorded at power and modulation amplitude settings which were previously determined to be below those causing saturation or line-width broadening. The spectra were analyzed with a Varian 620/L-100 computer. The experimental plots of the hyperfine splitting parameter ($2T_{||}$) vs. temperature were analyzed in terms of linear components by fitting regression lines to appropriate sections using the method of least squares. This empirical method of analysis has been previously used in the interpretation of results obtained from ESR experiments on synthetic lipid dispersions (Shimshick & McConnell, 1973) and prokaryotes (Weller & Haug, 1977; Yang & Haug, 1979), as well as B. emersonii (Leonards & Haug, 1980a, b). The temperatures at which break points are observed, as indicated by the intersections of straight lines, are in good agreement with those obtained by other physical-chemical techniques (Shimshick & McConnell, 1973; Lee, 1975). The break points obtained with this form of analysis were confirmed with a second analytical method, an iterative least squares program (Brunder, et al, unpublished) using normalized ß-splines (Dierckx, 1975).

Results

The middle break point, T_M, originally observed with electron spin resonance spectroscopy for zoospores *in vivo*, spin labeled with 5-nitroxystearate (5-NS) or 2,2,6,6-tetramethylpiperdine-1-oxyl (TEMPO) was markedly affected by the external Ca^{2+} ion concentration. Specifically, Ca^{2+} ions increased the value of T_M from 12 ± 1°C (no Ca^{2+} added) to 22 ± 1°C (10 mM $CaCl_2$ present), which was the growth temperature (Leonards & Haug, 1980a).

To ascertain if T_M was the result of a cation modulated lipid/lipid or lipid/protein interaction the zoospore plasma membrane was isolated, spin labeled with 5-NS, and the hyperfine splitting parameter, $2T_{||}$, measured as a function of temperature (Figure 1). A break point was observed at 21 ± 1°C for the membrane preparation, which was isolated and suspended in a buffered 10 mM $CaCl_2$ solution pH 6.7. The lipids were then extracted from the preparation, non-lipid contaminants removed (including more than 99% of the proteins), and aqueous dispersions of the membrane lipids re-examined, in the presence of 10 mM $CaCl_2$ (pH 6.7), as a function of temperature. The results obtained (Figure 1) were the same as those found for the plasma membrane preparations, indicating that the break point observed at 21 ± 1°C in the presence of 10 mM $CaCl_2$ was a

Figure 1 Plots of $2T_{||}$ vs. temperature for zoospore plasma membrane preparations (□—□) and aqueous dispersions of the plasma membrane lipids (○—○) spin labeled with 5-nitroxy-stearate (5-NS) and measured in the presence of 10 mM $CaCl_2$ (pH 6.7).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$2T_{II}$ (GAUSS)
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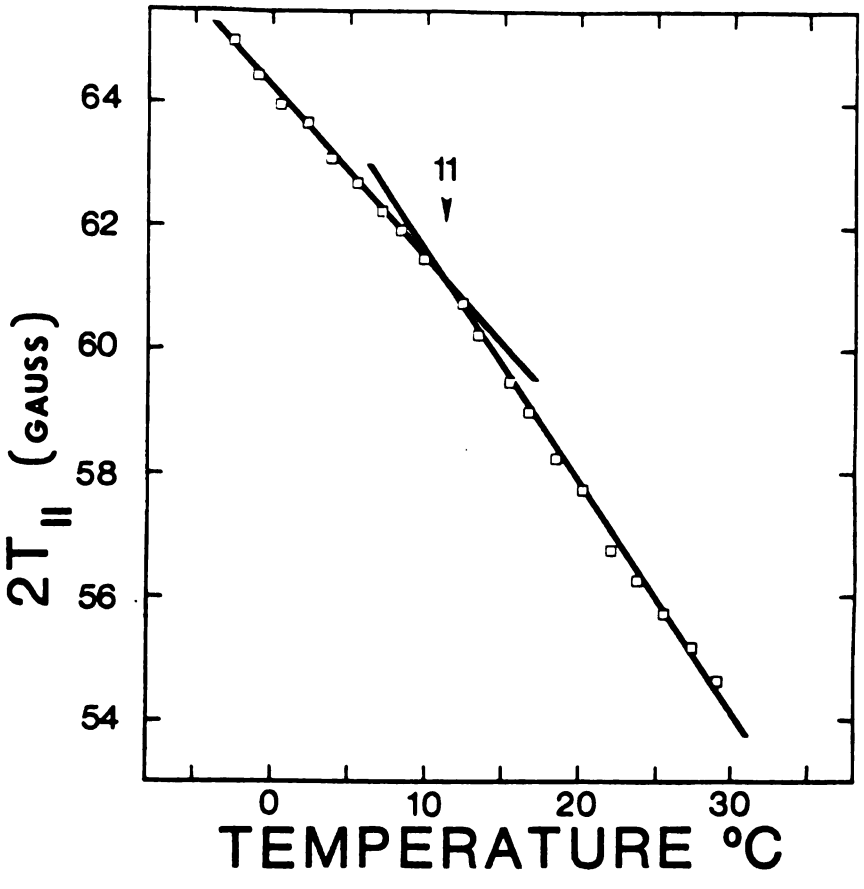
lipid/lipid interaction. Control experiments, using lipids extracted from membrane preparations which had not been previously used for ESR experiments gave the same result.

Although the Ca^{2+} ion effect involved lipid/lipid interactions the possibility existed that the T_M value obtained in the absence of Ca^{2+} ions was due to a lipid/protein interaction, which dissociated in the presence of Ca^{2+} ions. This possibility was examined by making spin labeled aqueous dispersions of the plasma membrane lipids and measuring $2T_{||}$ as a function of temperature in the presence of 10 mM $CaCl_2$ + 10 mM EDTA (pH 6.7). A break point was observed at 11 ± 1°C (Figure 2), indicating that the middle break point obtained in the absence of free Ca^{2+} ions was also due to a lipid/lipid interaction, and that the Ca^{2+} ion effect could be reversed by EDTA.

The initial ESR experiments with spin labeled zoospores indicated that K^+ ions could reverse the effects of Ca^{2+} ions, downshifting T_M from 22 ± 1°C to 10 ± 1°C (Leonards & Haug, 1979a; 1980a). The ability of K^+ ions to reverse the effects of Ca^{2+} ions in the isolated plasma membrane preparations was tested by first measuring the hyperfine splitting parameter, $2T_{||}$, of a spin labeled membrane sample in the presence of a buffered 10 mM $CaCl_2$ solution (pH 6.7)

Figure 2 Plot of $2T_{||}$ vs. temperature for aqueous dispersions of the plasma membrane lipids (□—□) spin labeled with 5-nitroxystearate (5-NS) and measured in the presence of 10 mM $CaCl_2$ + 10 mM EDTA (pH 6.7) demonstrating the reversal of the Ca^{2+} effect by EDTA.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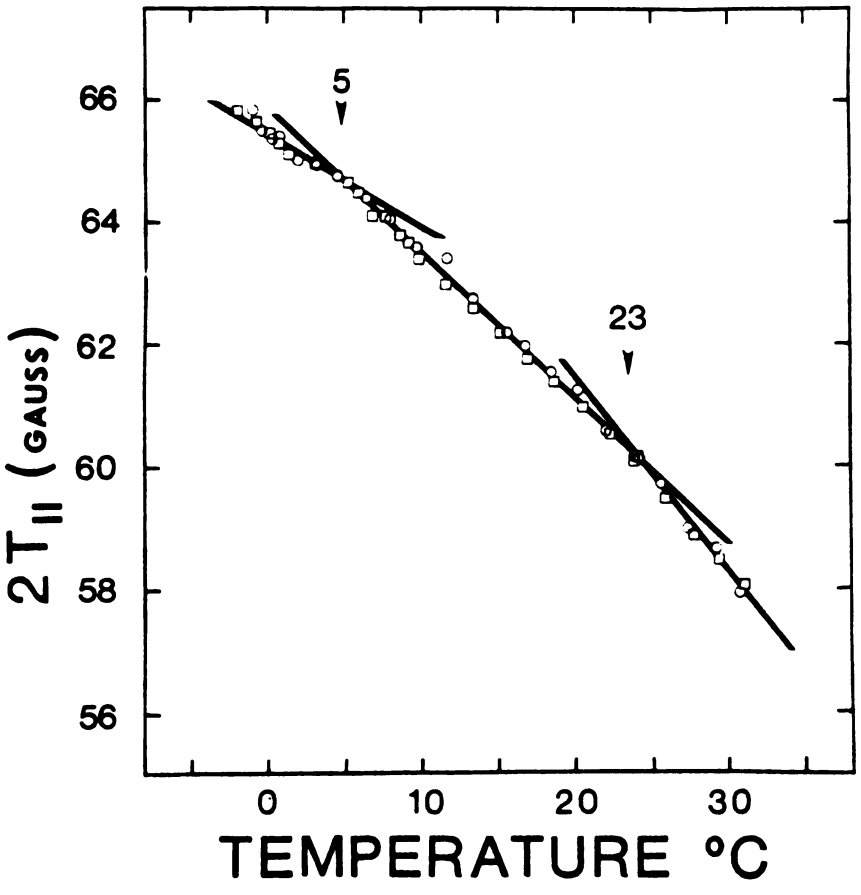
as a function of temperature, and then re-running the same sample after adding KCl (final concentration 50 mM) to the suspension. The results are illustrated in Figure 3. Two break points were observed in the presence of 10 mM $CaCl_2$ over the temperature range of 3-32°C. The first occurred at 5 ± 1°C and the second at 23 ± 1°C. These values are the same as the values obtained for T_L and T_M with 5-NS spin labeled zoospores under the same conditions (5 ± 1°C and 22 ± 1°C, respectively) (Leonards & Haug, 1980a). Addition of K^+ ions had no affect on T_L or T_M, the results obtained being indistinguishable from those observed with Ca^{2+} ions only (Figure 3). The absence of any change in T_L, as a consequence of cation addition, is in accord with previous results obtained for spin labeled zoospores (Leonards & Haug, 1979a; 1980a) and with the isolated lipid components from zoospores (Leonards & Haug, 1979b, 1980b). In contrast, the inability of K^+ ions to downshift T_M was inconsistent with the results of spin labeled zoospore experiments, under seemingly similar conditions.

A major difference between the experiments with the spin labeled zoospores and with the isolated plasma membranes was the absence of an energy source in the latter. Metabolically, the zoospores are quite active, expending energy derived from endogenous reserves, to

Figure 3 Plots of $2T_{||}$ vs. temperature for zoospore plasma membrane preparations spin labeled with 5-nitroxystearate (5-NS) indicating the inability of K^+ ions to reverse the Ca^{2+} ion effect in the isolated membranes. Plasma membranes in the presence of 10 mM $CaCl_2$ (pH 6.7) (O—O). Plasma membrane sample after KCl (final conc. 50 mM) was added to the preparation containing 10 mM $CaCl_2$ to reverse the Ca^{2+} effect (□—□).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maintain an osmotic balance with the medium, since the zoospore lacks a cell wall, and to provide energy for motility (Suberkropp & Cantino, 1972; 1973). In addition, living cells possess an electrochemical gradient across their plasma membranes, a condition which did not hold for the isolated plasma membrane preparations. The importance of such a gradient is supported by experiments which indicate that K^+ ions change the membrane potential of B. emersonii zoospores, and that these changes may be involved in the initial events of zoospore encystment (Jen & Haug, unpublished). Previous studies have also demonstrated that a transmembrane electrical potential ($\Delta\psi$) can be generated by Neurospora plasma membrane vesicles in the presence of added ATP (Stroobant & Scarborough, 1979).

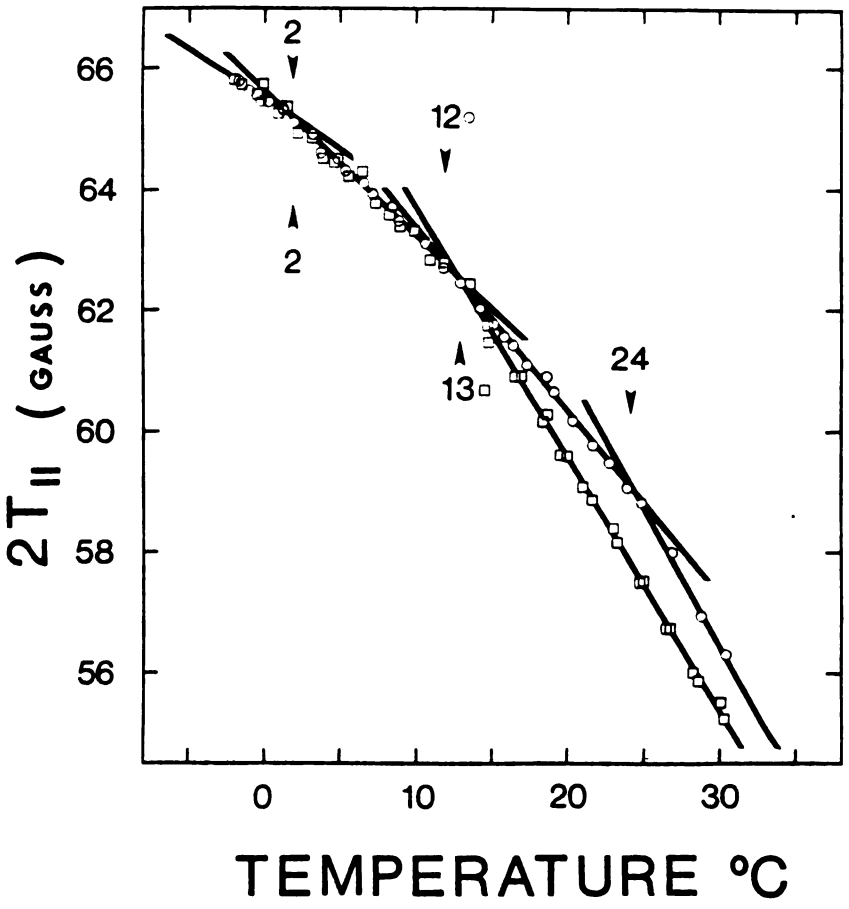
In order to determine if the K^+ ion induced reversal of the Ca^{2+} ion effect was related to an energy dependent process, freshly prepared ATP was added to the isolated plasma membrane preparation immediately before the initial sonication step (step a., materials and methods). If the reversal required the presence of an energized membrane, and this requirement could be fulfilled by ATP, a break point should be observed at about $10^{o}C$. The results obtained for membrane samples in the presence of 5 mM ATP + 10 mM $CaCl_2$ + 50 mM KCl (pH 6.7), measured as a function of temperature, are

illustrated in Figure 4. Three break points were observed, the lowest one (2 ± 1°C) being similar to the value previously noted for T_L. The second and third break points occurred at 12 ± 1°C and 24 ± 1°C. The 24 ± 1°C break is the same as that observed in Figure 3. The 12 ± 1°C break is virtually the same as that hypothesized above for K^+ ion reversal of the Ca^{2+} ion effect. The presence of both breaks is what one would expect for a membrane preparation composed of a mixture of right side out and inverted vesicles, and/or a mixture of sealed and leaky vesicles. Both types of ATP used gave the same results.

To further investigate the possibility that the membrane preparation was composed of such a mixed population of vesicles, buffered EDTA was added to the preparation (final conc. 10 mM) and the hyperfine splitting value, $2T_{||}$, re-measured as a function of temperature. These results are also shown in Figure 4. A break point was observed at 13 ± 1°C, but the break point previously observed at 24 ± 1°C was absent. The $2T_{||}$ values obtained up to 13°C were also indistinguishable from those observed before EDTA addition, confirming the break point at 12 ± 1°C.

To eliminate the possibility that the downshift in T_M observed in the presence of ATP was the result of an ATP chelation effect, control experiments with aqueous

Figure 4 Plots of $2T_{||}$ vs. temperature for zoospore plasma membrane preparations spin labeled with 5-nitroxystearate (5-NS) in the presence of 10 mM $CaCl_2$ + 50 mM KCl + 5 mM ATP (pH 6.7) (O—O) and 10 mM $CaCl_2$ + 50 mM KCl + 5 mM ATP + 10 mM EDTA (pH 6.7) (□—□), indicating that K^+ ions can reverse the Ca^{2+} ion effect in the plasma membrane samples if ATP is included. The sample was re-examined after EDTA addition to confirm the effect on T_M of Ca^{2+} removal.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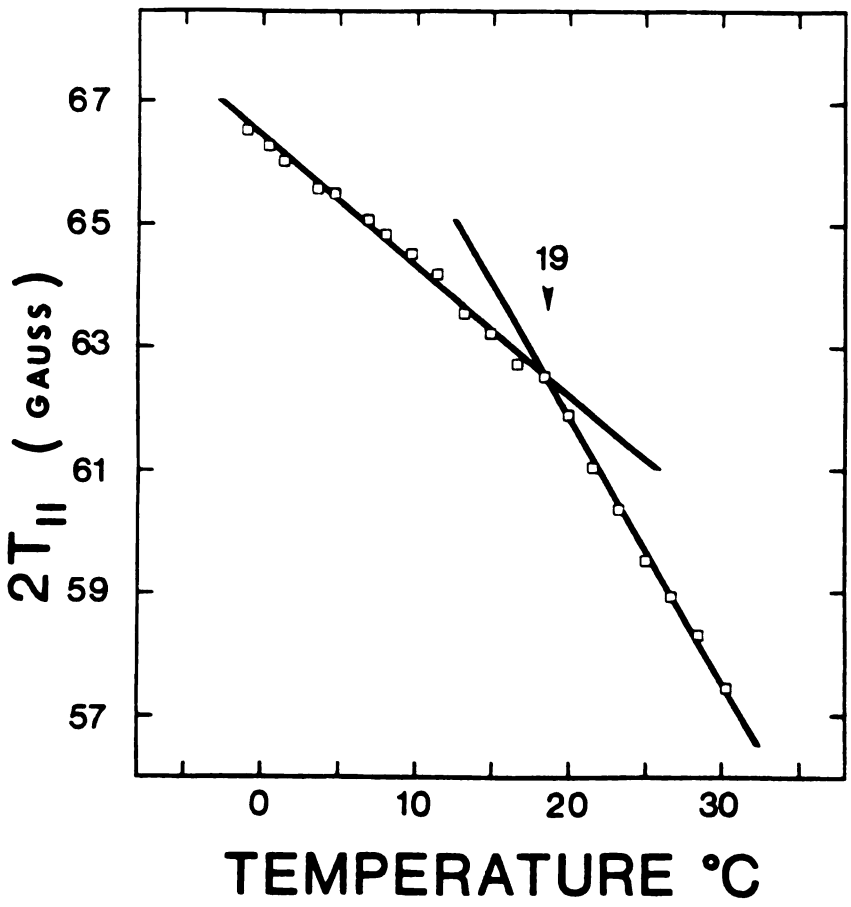
dispersions of the membrane lipid extracts (proteins removed) were examined as a function of temperature in the presence of buffered 5 mM ATP + 10 mM $CaCl_2$ + 50 mM KCl (pH 6.7). The results obtained were the same as those previously observed for aqueous dispersions of the membrane lipid extracts in the presence of 10 mM $CaCl_2$ (Figure 1), indicating that the downshift in T_M by ATP was not due to a chelation effect.

To obtain further information concerning the "energized membrane" requirement, and as another control experiment, ATP was replaced with its structural analogue AMP-PNP in the membrane preparations. Although AMP-PNP is structurally similar to ATP, it cannot be used as an energy source by most organisms. The results obtained for membrane samples in the presence of 5 mM AMP-PNP + 10 mM $CaCl_2$ + 50 mM KCl (pH 6.7) are illustrated in Figure 5. Only one break point was observed, at 19 ± 1°C, indicating that AMP-PNP could not substitute for ATP. Why the break point was downshifted from 24 ± 1°C to 19 ± 1°C, however, is not known at this time.

Figure 5 Plot of $2T_{||}$ vs. temperature for zoospore plasma membrane preparation spin labeled with 5-nitroxystearate (5-NS) in the presence of 10 mM $CaCl_2$ + 50 mM KCl + 5 mM AMP-PNP (pH 6.7) (□—□), indicating that AMP-PNP, a structural analogue of ATP, cannot substitute for ATP.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Discussion

Analysis of the hyperfine splitting parameter, $2T_{||}$, obtained for spin labeled plasma membrane preparations and aqueous dispersions of the plasma membrane lipid extracts indicates that the middle break point, T_M, found in the zoospore plasma membrane samples is the result of a lipid/lipid interaction rather than a lipid/protein interaction. The values obtained for T_M in the plasma membrane preparations are also the same as those observed in the ESR studies of spin labeled zoospores in vivo (Leonards & Haug, 1979a; 1980a) indicating that the lipid/lipid interaction found in the plasma membrane preparations is also responsible for the middle break point found in zoospores in vivo.

The possibility that the middle break point, T_M, represents the gel-to-liquid-crystalline phase transition of a bulk lipid phase is unlikely. The lower, T_L, and upper, T_H, break points have been observed in experiments with spin labeled zoospores in vivo (Leonards & Haug, 1979a; 1980a), zoospore plasma membrane preparations (unpublished data), aqueous dispersions of the total lipids extracted from zoospores (Leonards & Haug, 1979a; 1980a), and aqueous dispersions of the glycolipid fraction isolated from zoospores (Leonards & Haug, 1979b; 1980b). However, the middle break point, T_M, has not been detected either in aqueous dispersions of the total

lipids extracted from zoospores, or in aqueous dispersions of any of the isolated lipid components.

The presence of Ca^{2+} ions markedly affected the middle break point observed in the plasma membrane lipid extract, T_M being shifted from 21 ± 1°C (10 mM $CaCl_2$) down to 11 ± 1°C (Ca^{2+} ions removed by EDTA). This change in T_M is the same as that obtained in the initial ESR studies on spin labeled zoospores in vivo, where a Ca^{2+} ion specific effect on T_M was observed (Leonards & Haug, 1979a; 1980a). Chemical analysis of the lipid components isolated from the zoospore plasma membrane has shown that this membrane is almost entirely composed of diglucosyldiglycerides (≈45% by wt.) and neutral lipids (≈43% by wt., no triglycerides) rather than phospholipids (≈12% by wt.). Of the phospholipids present approximately 90% are phosphatidylcholine and phosphatidylethanolamine (Leonards & Haug, 1980c). In addition, experiments with aqueous dispersions of the lipid components isolated from the zoospores have shown that the zoospore's glycolipids interact strongly with Ca^{2+} ions, whereas Ca^{2+} ions have no detectable affect on the zoospore's phospholipid fraction (Leonards & Haug, 1979b; 1980b). The lipid/lipid interactions observed therefore cannot be attributed to the properties of the phospholipids. Since the middle break point, T_M, is the result of a lipid/lipid interaction which

can be influenced by Ca^{2+} ions, but cannot be attributed either to a lipid phase transition or the zoospores phospholipid fraction, the most plausible explanation is a glycolipid/glycolipid or glycolipid/neutral lipid interaction.

The specific molecular nature of a Ca^{2+} ion/glycolipid interaction is still unknown but certainly involves the lipid head-group. Because neither the glycolipids or neutral lipids (excluding free fatty acids) are charged molecules an interaction analagous to that induced by Ca^{2+} ions in phosphatidylserine or phosphatidic acid containing mixtures is unlikely. However the interaction of Ca^{2+} ions with glycolipids has been shown to increase the hydration capacity of diglucosyldiglyceride head-groups (Wieslander et al, 1978), possibly by altering the orientation of the glycolipid head-group. Hydration related head-group orientation changes have also been reported for thin films of phospholipid membranes (Jendrasiak & Mendible, 1976). A Ca^{2+} ion-induced glycolipid head-group change is also consistent with the results obtained with aqueous dispersions of zoospore glycolipids (Leonards & Haug, 1979b; 1980b).

The addition of K^{+} ions to the isolated plasma membrane preparations, in the presence of Ca^{2+} ions had no affect on the middle break point, T_M, when ATP was omitted. The absence of any change in the plots of

$2T_{||}$ vs. temperature for these preparations, after K^+ ion addition indicates that K^+ ions do not reverse the Ca^{2+} ion effect simply by replacing the Ca^{2+} ions. That is, K^+ ions and Ca^{2+} ions do not have the same interaction site. This conclusion is supported by the results obtained for aqueous dispersions of the zoospore glycolipids, which demonstrated that an increase in $2T_{||}$ observed as a consequence of Ca^{2+} ion inclusion could neither be prevented or reversed by the addition of K^+ ions (Leonards & Haug, 1980b).

A comparison of the results illustrated in Figure 4 to those in Figure 2 indicates that the temperature to which T_M is shifted by K^+ ions + ATP and EDTA in the isolated plasma membrane preparations, is the same as that found for the aqueous dispersions of the plasma membrane lipid extracts in the presence of EDTA (12 ± 1°C and 11 ± 1°C, respectively). This value for T_M is also similar to the T_M values obtained for the K^+ ion reversal of the Ca^{2+} ion effect in spin labeled zoospores <u>in vivo</u>, 10 ± 1°C (Leonards & Haug, 1979a, 1980a). These results indicate that the Ca^{2+} ion effect could be reversed in at least a portion of the vesicle preparation by K^+ ions, after adding ATP. This conclusion was confirmed by the data obtained after EDTA addition, which demonstrated that the removal of Ca^{2+} ions resulted in the downshifting of T_M to

13 ± 1°C, and eliminated the break point at 24 ± 1°C (Figure 4). These results suggest that the membrane proteins do not significantly affect the observed interaction. However, the mechanism of ATP action was not the same as that observed for EDTA, since the control experiments indicated that ATP was not downshifting T_M by a chelation effect, and AMP-PNP could not substitute for ATP.

The analysis of the results obtained before and after the inclusion of ATP in the plasma membrane preparations suggests that the ATP is being utilized to generate an "energized membrane". This suggestion was directly confirmed by experiments in our laboratory which demonstrated that a membrane potential was generated in zoospore plasma membrane preparations when ATP was added to the sample. Without the addition of ATP no such membrane potential existed. In addition, Cyclic-AMP could not substitute for ATP (Jen & Haug, unpublished). Such an interpretation is also consistent with the studies on Neurospora plasma membrane vesicles which indicated that a transmembrane electrical potential ($\Delta\psi$) could be generated in the presence of ATP (Stroobant & Scarborough, 1979).

Given that ATP can be used to generate a membrane potential in zoospore plasma membrane preparations, and that once an "energized membrane" state is achieved

K^+ ions can reverse the Ca^{2+} ion effect, causing a downshift in T_M, what is the connection between these phenomenon and T_M? Specifically, how is a change in a lipid/lipid interaction, which can be accomplished by EDTA and Ca^{2+} ions in lipid extracts and involves the lipid head-group, related to the generation of an "energized membrane" by ATP, which requires the presence of membrane proteins?

Since K^+ ions do not simply replace Ca^{2+} ions, the reversal of the Ca^{2+} ion effect must involve other membrane parameters. Besides the downshift in T_M, the addition of K^+ ions has been shown to result in the immediate release of $^{45}Ca^{2+}$ from preloaded zoospores (Soll & Sonneborn, 1972). K^+ ion addition also causes a depolarization of the membrane potential, both in zoospores <u>in vivo</u> and in plasma membrane preparations after a membrane potential was first generated by ATP addition (Jen & Haug, unpublished). In addition, experiments with lipid membrane vesicles have demonstrated that a transmembrane potential interacts strongly with phosphatidylserine and phosphatidylcholine head-groups, alterations in the orientation of the surface dipoles of the lipid molecules being caused by different membrane potentials (Lelkes, 1979). Together these results suggest that the reversal of the Ca^{2+} ion effect, and thus the downshift in T_M, is a consequence

of the K^+ ion induced depolarization of the membrane potential. This hypothesis is consistent with the effects of K^+ ions on the membrane potential, which explains the requirement for an "energized membrane", and at the same time indicates how K^+ ions could reverse the Ca^{2+} ion effect, altering a lipid/lipid interaction. It should be noted that this hypothesis does not exclude other consequences of either K^+ ion addition or the depolarization of the membrane potential on cellular functions. Nor does this hypothesis indicate a specific molecular mechanism by which K^+ ions cause a depolarization of the membrane potential.

The physiological significance of the middle break point is that it is closely correlated with zoospore differentiation. The lipid/lipid interactions observed, as T_M, in the plasma membrane seem to be intimately involved with the temperature dependence of zoospore encystment. Ca^{2+} ions change this temperature dependence by interacting directly with the lipid molecules involved. In contrast, K^+ ions exert their influence on T_M indirectly. If the hypothesis that K^+ ions reverse the Ca^{2+} effect, downshifting T_M via a depolarization of the membrane potential is correct, it indicates that lipid/lipid interactions in a plasma membrane can be altered by changes in a transmembrane electrical potential. The regulation of zoospore

differentiation may, therefore, involve both electrical and structural properties of the zoospore plasma membrane.

Acknowledgements

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GENERAL CONCLUSIONS

Physiologically, the initial changes observed during zoospore encystment and germination involve the plasma membrane. To gain information on the molecular alterations at the cell surface associated with these changes we examined the physical-chemical properties of the zoospore plasma membrane *in vivo* and in isolated plasma membrane preparations. We specifically concerned ourselves with the properties of the lipid matrix of the membrane and therefore also examined the total lipids and isolated lipid components extracted from zoospores and the isolated zoospore plasma membrane.

The first section of this dissertation established a correlation between the physiological effects of temperature and cations on zoospore viability and differentiation, and their physical-chemical effects on the dynamic properties of the plasma membranes of intact zoospores. Intact zoospores and zoospore total lipid extracts were spin labeled with 5-nitroxystearate (5-NS), 12-nitroxystearate (12-NS), and 2,2,6,6-tetramethylpiperdine-1-oxyl (TEMPO). The initial experiments indicated that 12-NS was inappropriate for this organism ($2T_{||}$ values could not be determined). However, the

ability to use both the 5-NS and TEMPO spin labels permitted us to probe the zoospore plasma membrane in two related, but significantly separate, ways. Electron spin resonance spectroscopy indicated a total of three breaks in plots of the hyperfine splitting parameter, $2T_{||}$, order parameter, S, and partition parameter, f, as a function of temperature. The first and third break points (T_L and T_H) were found to be independent of the external K^+, Ca^{2+}, or Mg^{2+} ion concentrations. They were similar to the break points found in aqueous dispersions of the lipid extracts and correlate well with the temperature limits for zoospore viability. In contrast, the middle break point, T_M, was markedly influenced by the external Ca^{2+} ion concentration. Ca^{2+} ions increased T_M from 12 ± 1°C (no Ca^{2+} added) to 22 ± 1°C (10 mM Ca^{2+}). Twenty-two degrees is just above growth temperature. Increasing the Ca^{2+} ion concentration to 20 mM did not further increase T_M, suggesting a saturation effect. K^+ ions reversed this Ca^{2+} ion effect, downshifting T_M from 22 ± 1°C to 10 ± 1°C. The physical-chemical effects of these ions on the membrane, as revealed by the cation-induced shifts in T_M, was found to be closely correlated with the temperature dependence and physiological effects of these cations on zoospore differentiation.

The second section of this dissertation expanded upon these initial results to determine which lipid components gave rise to the phase transformations previously observed, and to indicate some of the general properties concerning the interactions of these components with other lipids and ions. The physical-chemical properties of the lipid components isolated from zoospores were investigated with ESR using the spin label 5-nitroxy-stearate (5-NS). Lipid dispersions were made from zoospore phospholipids and glycolipids, both singly and in combination with each other and with isolated neutral lipid components.

Plots of the hyperfine splitting parameter ($2T_{||}$) vs. temperature indicate that it is the zoospore glycolipids rather than the phospholipids which are responsible for the phase transformations previously observed in aqueous dispersions of the total lipids extracted from zoospores and in zoospores <u>in vivo</u>. The discontinuities observed in the glycolipid dispersions seem to represent the onset and completion of a gel-to-liquid-crystalline phase transition. Over the temperature range tested, calcium ions increased the rigidity of the glycolipid dispersions, the major component of which is probably a diglucosyldiglyceride, but had no effect on the phospholipid dispersions. This increase in $2T_{||}$ was not affected by inclusion of neutral lipids

into the glycolipid dispersion but was eliminated at high (5 to 1 wt./wt.) phospholipid to glycolipid ratios. The Ca^{2+} effect was relatively independent of both the absolute rigidity of the dispersion and its phase (gel or liquid-crystalline), suggesting an interaction with the glycolipid head-group rather than the hydrocarbon core. The Ca^{2+} ion-induced increase in $2T_{||}$ was neither prevented nor reversed by the presence of K^{+} ions.

The presence of two spin label populations co-existing in a dynamic equilibrium was found in glycolipid/neutral lipid dispersions. Plots of the percentage ($\{H_A/(H_A + H_B)\} \times 100$) of the spin label population, as measured by the peak height of the low field peaks, corresponding to the more immobilized component (H_A) vs. temperature indicated two break points. The temperatures at which these break points occurred are similar to those obtained for the glycolipid dispersions, and match the break points (T_L and T_H) found in ESR experiments using zoospores in vivo.

Although the lower and upper break points (T_L and T_H) had been observed in the glycolipid fraction isolated from zoospores, the middle break point (T_M) had only been observed in the studies with spin labeled zoospores in vivo. In addition, the finding that T_L and T_H were attributed to the glycolipids, and

not the phospholipids, implied that the plasma membrane of B. emersonii zoospores must contain a significant percentage of glycolipid. To further examine these questions required a plasma membrane preparation.

The third section of this dissertation described a procedure for the isolation of zoospore plasma membranes, which involved the rupturing of the plasma membrane. The ultrastructural organization of the zoospore then permitted the removal of the major potential membrane contaminants as a single unit.

Analysis of the membrane lipids indicated that the zoospore plasma membrane was composed of glycolipids and neutral lipids, rather than phospholipids. Moreover, the glycolipid fraction contained only diglycosyldiglycerides, with 90% of the glycolipid head-group sugars being glucose. These results indicated that the single largest group of lipids present in the zoospore plasma membrane (≃47% of total) were diglucosyldiglycerides. The plasma membranes were also characterized by an absence of triglycerides.

The preponderance of diglucosyldiglycerides in the plasma membrane was the evidence necessary to unite the first two sections of this dissertation and directly indicated a role for the glycolipids in the encystment process.

The purpose of the fourth section of this

dissertation was to specifically study the middle break point, T_M, in isolated plasma membrane preparations to determine 1.) if T_M was the result of a lipid/protein or lipid/lipid interaction, and 2.) to further examine the effects of cations on T_M. Both isolated plasma membranes and aqueous dispersions of the lipids extracted from the plasma membranes were spin labeled with 5-nitroxystearate and analyzed as a function of temperature with ESR spectroscopy.

Plots of the hyperfine splitting parameter ($2T_{||}$) vs. temperature indicated that the middle break point, T_M, was the result of a lipid/lipid interaction (glycolipid/glycolipid or glycolipid/neutral lipid) rather than a lipid/protein interaction. This interaction was markedly affected by Ca^{2+} ions, which interacted directly with the lipid components, increasing T_M from 11 ± 1°C to 21 ± 1°C in the lipid dispersions and 12 ± 1°C to 23 ± 1°C in the plasma membrane preparations.

The initial ESR studies on spin labeled zoospores <u>in vivo</u> had also demonstrated that the addition of K^+ ions could reverse the Ca^{2+} ion effect, downshifting T_M from 22 ± 1°C to 10 ± 1°C. The addition of K^+ ions to the isolated plasma membrane had no affect on T_M, indicating that K^+ ions do not simply replace Ca^{2+} ions, but exert their effect indirectly on the membrane.

However, after the inclusion of ATP, K^+ ions could reverse the Ca^{2+} ion effect. It was determined that the ATP generated an "energized membrane" state which permitted the K^+ ions to reverse the Ca^{2+} effect. Since K^+ ions have been shown to depolarize the membrane potential in both zoospores and isolated zoospore plasma membrane preparations (generated by ATP), we suggest that the K^+ ion-induced reversal of the Ca^{2+} ion effect, and therefore the change in the lipid/lipid interactions responsible for T_M, is a consequence of the K^+ ion-induced depolarization of the membrane potential.

Together the four sections of this dissertation indicate that the physical-chemical properties of the zoospore plasma membrane are intimately related to the differentiation process in this organism. Three phenomenon observed in this study have not been previously reported in eukaryotes.

The first is the presence of three break points in plots of $2T_{||}$, S, or f vs. temperature rather than the characteristic two breaks. Whether the presence of a middle break point is common in differentiating eukarytoic cells, or is unique for B. emersonii zoospores is presently unknown. However, the high degree to which T_M is related to zoospore differentiation, suggests that similar phenomenon may be observed in other fungi having a similar zoospore stage in their

life cycle.

The second phenomenon previously unreported for a eukaryote is the existence of a glycolipid rather than a phospholipid based plasma membrane lipid matrix. Although glycolipid rich membranes have been found in A. laidlawii (Weiselander et al, 1978), plasma membranes have been largely characterized as phospholipid structures. Since the processes normally associated with phospholipid membranes also occur in glycolipid membranes, i.e., membrane fusion, aggregation, or adhesion, any general mechanism proposed to explain the molecular basis of these processes must be applicable to both types of plasma membranes.

The third phenomenon previously unreported is the correlation between an energized membrane state, and cation induced changes in lipid/lipid interactions within a plasma membrane. The significance of this correlation rests upon the relationship between T_M and zoospore differentiation. If this correlation can be generalized to other differentiating organisms, it indicates a molecular meeting point between three previously unrelated phenomenon which may be basic to the regulation of the differentiation process.

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