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

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Summary

The physico-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 ($2T_{\parallel}$) 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. The increase in $2T_{\parallel}$ was not affected by inclusion of neutral lipids into the glycolipid dispersion but was eliminated at high (5 : 1, w/w) 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+}-induced increase in $2T_{\parallel}$ was neither prevented nor reversed by the presence of K^{+}.

The presence of two spin label populations co-existing in a dynamic equi-

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; TLC, Thin-layer chromatography; GLC, Gas-liquid chromatography; 5-nitroxystearate, 2-(3-carboxypropyl)-4, 4-dimethyl-2-tridecyl-3-oxazolidinyloxyl.

librium 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.

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

Introduction

The aquatic phycomycete, *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 physico-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 *B. 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 Mops plus 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 × *g* for 5 min 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—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 acid methyl esters were separated by gas-liquid chromatography at 170°C on a 2 m × 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 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-nitroxy-stearate, was used for these ESR studies. Aqueous dispersions of the lipid components were made by mixing aliquots 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 N_2 and sonicated (125 W 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—10 min to incorporate the label. The concentration of the spin label was less than 0.2% (w/w). Control experiments indicated that the small amounts of ethanol added did not alter the spectra measured. For some experiments ethylene glycol (final concentration 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 $2T_{\parallel}$ vs. temperature [11]. In experiments to test the effects of Ca^{2+} on membrane rigidity, $CaCl_2$ (final concentration 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+} 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 ($2T_{\parallel}$), 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 Fig. 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, $2T_{\parallel}$, is indicative of motional properties of the spin probe in the membrane. A high value of $2T_{\parallel}$ is associated with restricted motion or increased rigidity and a low $2T_{\parallel}$ value corresponds 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 physicochemical techniques [16—18].

Results

Lipids—analytical studies

The percentage of neutral, glyco- and phospholipid fractions found in zoospores were, respectively, 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

TABLE I

FATTY ACYL COMPOSITION OF TOTAL LIPIDS, PHOSPHOLIPIDS AND GLYCOLIPIDS OF *B. EMERSONII* ZOOSPORES

Fatty acyl composition determined as the methyl esters by gas-liquid chromatographic analysis. Values are averages of multiple experiments ± S.E.; number of experiments indicated in parentheses. 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. —, values less than 1%.

Lipid	Fatty acyl composition (mol%)								Unsaturated/ saturated fatty acyl (mol/mol)
	16:0	16:1	18:0	18:1	18:2	γ-18:3	20:0	20:4	
Total lipid (4)	27.8 ± 0.25	—	2.6 ± 0.1	17.9 ± 0.3	5.2 ± 0.2	16.5 ± 0.25	1.1 ± 0.1	28.9 ± 0.85	2.2/1
Phospholipid (5)	19.7 ± 0.3	1.3 ± 0.02	—	5.6 ± 0.15	5.4 ± 0.15	23.7 ± 0.3	3.0 ± 0.3	40.6 ± 0.65	3.3/1
Glycolipid (4)	12.0 ± 0.05	1.0 ± 0.1	—	14.6 ± 0.15	6.2 ± 0.3	34.2 ± 0.3	8.3 ± 0.25	23.4 ± 0.85	3.8/1
Neutral lipid (4)	18.6 ± 0.15	2.5 ± 0.1	5.8 ± 0.2	25.9 ± 0.9	4.1 ± 0.1	9.9 ± 0.3	1.2 ± 0.1	31.8 ± 0.75	

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 system, 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 analyzed as a function of temperature (Fig. 1). A break point was observed in the glycolipid dispersions at 33 ± 1°C. A second break point was

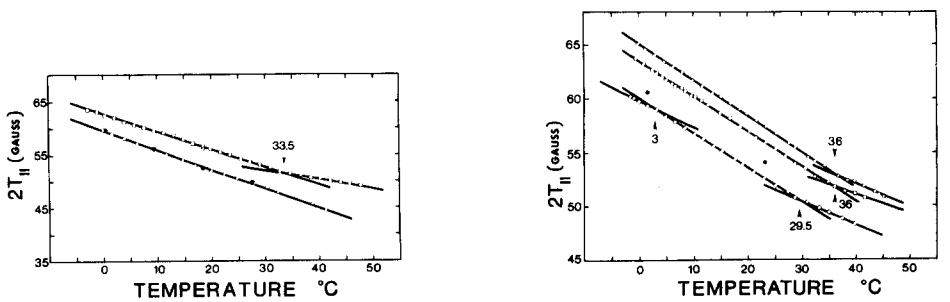

Fig. 1. Plot of $2T_{\parallel}$ vs. temperature for phospholipid and glycolipid dispersions labeled with 5-nitroxystearate. □———□, glycolipids; ○———○, phospholipids; ●———●, phospholipids + 20 mM $CaCl_2$.

Fig. 2. Plot of $2T_{\parallel}$ vs. temperature for mixed lipid dispersions labeled with 5-nitroxystearate. ◇———◇, glycolipids/phospholipids (1 : 1, w/w); ●———●, glycolipids/phospholipids (1 : 1, w/w) + 10 mM $CaCl_2$; □———□, glycolipids/phospholipids/cholesterol (1 : 1 : 1, w/w/w); ○———○, glycolipids/phospholipids/cholesterol (1 : 1 : 1, w/w/w) + 10 mM $CaCl_2$. In neither case did the addition of Ca^{2+} change the temperature at which T_H occurred.

observed at 2 ± 1°C with glycolipid dispersions in the presence of ethylene glycol when measured over the temperature range —12 to 20°C (data not shown). These break points are essentially the same as those found in plots of $2T_{\parallel}$ 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, in vivo, 5 ± 1°C and 33 ± 1°C, respectively [11]. No break point was 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-nitroxystearate 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 I) and the phase transition temperatures of phosphatidylcholine liposomes containing unsaturated fatty acyl chains [19,20]. This difference in membrane fluidity varied with temperature (Fig. 1), the glycolipid dispersions being characterized by $2T_{\parallel}$ values 3 G greater than the phospholipid dispersions at 0°C, and about 4 G greater at 22°C (growth temperature). This difference increased above the 33 ± 1°C breakpoint to 5—6 G.

The presence of 10 mM $CaCl_2$ did not change the $2T_{\parallel}$ values obtained with the phospholipid dispersions (Fig. 1). Glycolipid dispersions, in contrast, were affected by Ca^{2+}, the membrane becoming 1.5—2.5 G more rigid at all temperatures (Table II, Fig. 2).

TABLE II

EFFECT OF LIPID COMPOSITION AND Ca^{2+} ON THE HYPERFINE SPLITTING PARAMETER ($2T_{\parallel}$) OBTAINED FOR AQUEOUS DISPERSIONS LABELED WITH 5-NITROXYSTEARATE AND MEASURED AT 23°C

Values are averages of three experiments. The standard deviations were ≤0.25 G. Resolution of any single $2T_{\parallel}$ value was ±0.1 G. The free fatty acid region found on the TLC plates was actually composed of three 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 from the free fatty acids, exposing two previously hidden components. The nature of these components is unknown, although they do contain fatty acyl chains. $+Ca^{2+}$, final concentration 10 mM$CaCl_2$.

Lipid composition (by weight)	$2T_{\parallel}$ (G)		
	$-Ca^{2+}$	$+Ca^{2+}$	ΔG
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 *	48.25	53.25	5.0
Glycolipid/free fatty acids *	55.25	57.25	2.0
Glycolipid/sterols *	58.5	59.5	1.0
Glycolipid/diglycerides *	55.25	56.75	1.5
Glycolipid/phospholipid/cholesterol (1:1:1)	56.0	57.5	1.5

* Component ratio same as existed in total lipid extracts.

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 (Fig. 2). At a glycolipid-to-phospholipid ratio of 1 : 1 (w/w) the upper breakpoint (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, w/w/w) the temperature range of the transition was significantly broadened, T_H occurring at 36 ± 1°C. The lower break point (T_L) was down-shifted 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 G increase in $2T_\parallel$ over the entire temperature range.

In the presence of 10 mM $CaCl_2$, the $2T_\parallel$ values obtained for both the glycolipid/phospholipid (1 : 1, w/w) and glycolipid/phospholipid/cholesterol (1 : 1 : 1, w/w/w) dispersions increased by 1—2 G. However, the addition of calcium did not change the temperature at which T_H occurred in either dispersion.

To characterize further the interactions possible between various lipid components, aqueous dispersions were made from specific lipids, spin-labeled, and the hyperfine splitting parameter ($2T_\parallel$) 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 $2T_\parallel$ value, indicating a relatively fluid membrane. Addition of $CaCl_2$ increased the $2T_\parallel$ value by 5 G. 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 $2T_\parallel$, where there was no effect on the more fluid phospholipid dispersion.

Aqueous dispersions of phospholipid/glycolipid mixtures at different lipid-to-lipid ratios had an intermediate $2T_\parallel$ value. At a phospholipid-to-glycolipid ratio of 5 : 1 (w/w), the addition of $CaCl_2$ did not increase the $2T_\parallel$ value obtained. In contrast, at a 1 : 1 (w/w) ratio, the presence of Ca^{2+} increased the $2T_\parallel$ value by 1.5 G, which is similar to that obtained for the pure glycolipid dispersions.

Mixtures of glycolipids and different neutral lipid fractions had variable $2T_\parallel$ values, with the glycolipid/sterol dispersion having the highest $2T_\parallel$ value of any sample tested, 58.5 G, and the glycolipid/triglyceride mixture the lowest, 48.25 G. Thus, there was more than a 10 Gauss difference in values between 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 $2T_\parallel$ (1—2 G). The difference in the $2T_\parallel$ values (Table II) for a glycolipid/neutral lipid (1 : 3) dispersion compared to those of the glycolipid dispersion was probably caused by the presence of sterols in the neutral lipids, since sterols markedly enhanced the $2T_\parallel$ 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 $2T_\parallel$ value obtained by about 2 G. This calcium effect could be reversed by EDTA. The addition of KCl did not reverse the calcium effect, nor could it inhibit it.

TABLE III

EFFECT OF Ca^{2+} AND K^+ ADDITION ON THE HYPERFINE SPLITTING PARAMETER ($2T_{\parallel}$) VALUES OBTAINED FOR GLYCOLIPID DISPERSIONS LABELED WITH 5-NITROXYSTEARATE, AND MEASURED AT 24°C (pH 6.7)

Sequence	$2T_{\parallel}$ (G)
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

To approximate the stoichiometry of the glycolipid-Ca^{2+} interaction, glycolipid dispersions were spin-labeled with 5-nitroxystearate and the hyperfine splitting parameter ($2T_{\parallel}$) measured as a function of increasing Ca^{2+} concentration. The results of such an experiment are shown in Fig. 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 Ca^{2+} present is interacting with the outer lipid monolayer, one arrives at a glycolipid/Ca^{2+} 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-nitroxystearate-labeled zoospores suggested the presence of two spin label populations characterized by different degrees of spin label immobilization (Fig. 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 whether the glycolipids were involved in these changes, aqueous dispersions of the zoospore glyco-

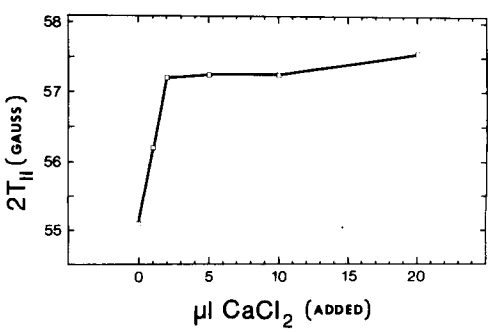

Fig. 3. Plot of $2T_{\parallel}$ vs. total amount (μl) of $CaCl_2$ added to glycolipid dispersions labeled with 5-nitroxystearate. Temperature 23°C; lipid concentration was 17 mg/ml; volume 0.5 ml; pH 6.7. Millimolarity of $CaCl_2$ solution is 2 × μl added.

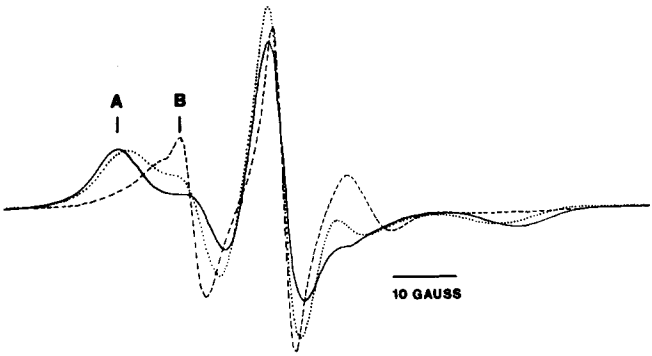

Fig. 4. Representative ESR spectra of 5-nitroxystearate-labeled zoospores recorded at different temperatures. The spectra are composed of two components, designated A and B. ———, 2°C; · · · · · ·, 9°C; - - - - - -, 36°C.

lipids 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 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, w/w), 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 (Fig. 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 phys-

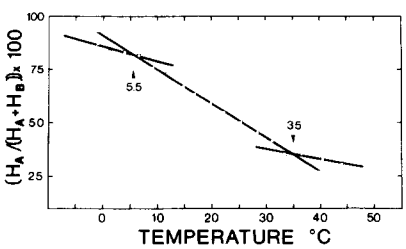

Fig. 5. Plot of $[H_A/(H_A + H_B)] \times 100$ vs. temperature of glycolipid/neutral lipid dispersions (1 : 3, w/w) labeled with 5-nitroxystearate, where H_A and H_B correspond to the peak heights of the low-field peaks.

ico-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, K.S. and Haug, A., unpublished results).

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 occur 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 (Fig. 4 and Ref. 11). This phenomenon was observed in lipid dispersions composed of glycolipids and neutral lipids, but was not present in dispersions of glycolipids alone. 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 (Fig. 5) may therefore represent the movements 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-nitroxystearate probe. Test experiments indicate that this is a valid assumption for this particular spin label. The temperature at which these break points occurred, 5 ± 1°C and 35 ± 1°C, are similar to the lower and upper (T_L and T_H) break points found with zoospores in vivo [11]. This suggests that the viability limits of the zoospore may be determined by the phase properties of the glycolipids.

The variability in the $2T_{\parallel}$ 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 $2T_{\parallel}$ observed for glycolipid/zoospore sterol dispersions, and the broadening of the temperatures 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 inflexibility of the planar ring structure of the sterol and the steric restrictions placed on adjacent fatty acyl chains.

The $2T_{\parallel}$ 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 $2T_{\parallel}$ 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.

Ca^{2+} increased the rigidity of the glycolipid dispersions as measured by the 5-nitroxystearate probe, but had no effect on the phospholipid dispersions (Fig. 1, Table II). These results are consistent with the analytical data (Results and Ref. 14) which demonstrated that the major phospholipids present were phosphatidylcholine and phosphatidylethanolamine, neither of which interact with Ca^{2+} at the concentrations used [24].

In contrast, glycolipids, especially diglucosyldiglycerides, have been shown to interact with Ca^{2+} [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+} on the sample, even though the base $2T_{\parallel}$ values were affected (Table II), suggests that Ca^{2+} interacts with the lipid head groups rather than the hydrocarbon core. In addition, the Ca^{2+}-induced increase in $2T_{\parallel}$ 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, these data are consistent with a Ca^{2+}-glycolipid head-group interaction.

The increase in $2T_{\parallel}$ seen after Ca^{2+} 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 $2T_{\parallel}$ vs. temperature (Fig. 2 and unpublished results). This result suggests that the effect of Ca^{2+} 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+} 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+} interaction was calculated to be 2.1 : 1 or 2.7 : 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+} present in the solution was interacting with the glycolipid molecules. A glycolipid-Ca^{2+} ratio of 4.4 : 1 has been previously reported to strongly affect the hydration capacity of a diglucosyldiglyceride [21]. Whether this ratio represents a mini-

mum or maximum number, however, was not stated.

There was little change in the $2T_{\parallel}$ values obtained for glycolipid dispersions in the presence of K^+. In addition, the presence of K^+ had no effect on the Ca^{2+}-induced increase in $2T_{\parallel}$ observed in the glycolipid dispersions (Table III). The inability of K^+ either to prevent or to 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+} on the physico-chemical properties of the zoospore plasma membrane could be reversed by the addition of K^+ (same ion concentrations) [11]. Other studies have also demonstrated an immediate release of $^{45}Ca^{2+}$ from zoospores upon K^+ addition [26]. Therefore, if the glycolipids were solely responsible for the Ca^{2+} effects observed in vivo, the reversal of these effects by K^+ must involve other membrane parameters, and not be due to the simple replacement of Ca^{2+} by K^+.

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 *N*-acetylglucosamine units [27]. These findings suggest that the physico-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 phosphatidic acid or phosphatidyl serine detected) indicates that any general model for membrane fusion proposed must also be applicable to glycolipid membranes.

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