Biochimica et Biophysica Acta, 552 (1979) 66—77

BBA 78296

ANALYSIS OF *DICTYOSTELIUM DISCOIDEUM* PLASMA MEMBRANE FLUIDITY BY ELECTRON SPIN RESONANCE

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(Received June 9th, 1978)

Key words: ESR; Fluidity analysis; (Dictyostelium discoideum membrane)

Summary

Dictyostelium discoideum grown axenically in media containing polyunsaturated fatty acids exhibited normal growth rates but impaired differentiation (Weeks, G. (1976) Biochim. Biophys. Acta 450, 21—32). Since cell-cell contact is vital for differentiation but unnecessary for growth we have examined the isolated plasma membranes of these cells. The lipids of the plasma membranes of cells grown in the presence of polyunsaturated fatty acids contain considerable quantities of these acids, but the total phospholipid and sterol contents of the plasma membrane are close to normal. Electron spin resonance studies using 5-doxyl-stearic acid as the spin probe reveal two things. Firstly, there are no detectable characteristic transition temperatures in the plasma membranes of *D. discoideum*. Secondly, the plasma membranes of cell grown in the presence of polyunsaturated fatty acids have essentially the same fluidity as that of the control cells. The possible significance of this result to impaired cell-cell interaction is discussed.

Introduction

The fatty acid composition of the cellular slime mould, *Dictyostelium discoideum* has been modified by growing cells in an axenic medium in the presence of the polyunsaturated fatty acids linoleic, linolenic and arachidonic acids, either alone or in combination [1]. Considerable quantities of the three acids were incorporated into the cellular lipids and linoleic and linolenic acids were also further desaturated [1]. The cells grew normally, but the subsequent differentiation process was delayed or impaired.

Abbreviations: PMSF, phenylmethylsulphonyl fluoride; TEMPO, 2,2,6,6-tetramethyl piperidine-1-oxyl.

Since cell-cell contact is obligatory for differentiation but unnecessary for growth, it was postulated that impaired differentiation was due to impaired cell-cell contact resulting from structural changes in the plasma membranes [1]. In order to ascertain whether plasma membrane changes have occurred in polyunsaturated fatty acid-supplemented cells, we have prepared highly purified plasma membranes, analyzed their fatty acid and sterol composition and determined their membrane fluidity by electron spin resonance (ESR). The results of this study are presented in this report.

Materials and Methods

Organism and growth conditions. D. discoideum, Ax-2 was grown in a rich nutrient medium containing varying amounts of linoleic, linolenic or arachidonic acid as described previously [1]. The cells were grown to a cell density of $6 \cdot 10^6$ cells/ml and were then harvested by centrifugation at $700 \times g$ for 10 min. The pelleted cells were resuspended in 8.6% (w/v) sucrose/5 mM Tris-HCl (pH 7.5), (sucrose/Tris buffer) and recentrifuged at $700 \times g$ for 10 min. This wash procedure was repeated.

Plasma membrane purification. Washed cells ($\approx 10^{10}$) were resuspended in sucrose/Tris buffer saturated with phenylmethylsulphonyl fluoride (PMSF) at a cell density of 10^8 cells/ml. The cells were homogenized by vigorous stirring in the presence of glass beads, and pure plasma membranes were isolated from these homogenates by isopycnic sucrose density centrifugation, as previously described [2].

Protein, lipid, fatty and sterol analysis. The protein content of the purified plasma membrane preparation was determined by the Folin procedure [3]. Lipid was extracted from the purified plasma membrane preparations by the method of Bligh and Dyer [4], as described previously [1]. Lipid phosphorus was determined by the method of Ames [5] and the amount of phospholipid was calculated assuming an average molecular weight of 730 for the phospholipid of *D. discoideum* [6,7].

The lipid fraction was saponified and the fatty acid composition was determined by previously described gas-liquid chromatographic procedures [2]. The sterol fraction was obtained from the nonsaponifiable material by thin-layer chromatography. A suitable aliquot of pentane extract was evaporated to dryness under N_2, and dissolved in a small volume of chloroform for application to a thin-layer plate of silica gel H. Plates were developed using two solvent mixtures [8]. A cholesterol standard was run at the edge of each plate and visualized with Rhodamine G spray in order to locate the sterol zone. This zone was removed and extracted three times with 5-ml portions of chloroform/methanol (3 : 1, v/v). The silica gel was removed by centrifugation and, following evaporation to dryness, samples were acetylated with acetic anhydride at 140°C for 30 min and analyzed directly by gas-liquid chromatography, as described previously [2]. Cholesterol was employed as an internal standard. The sterol content determined by this procedure was identical to that obtained by the digitonide precipitation procedure described earlier [2].

In other experiments, the plasma membranes were saponified directly with 15% methanolic KOH prior to fatty acid and sterol analysis. The fatty acid

compositions and sterol contents were identical to those determined following lipid extraction and saponification of the lipid extract.

Preparation of samples for ESR. Washed cells were resuspended in 2 ml of 5 mM Tris-HCl, pH 7.4 (approx. $5 \cdot 10^7$ cells/ml) and 1 μl of methanolic solution of the doxyl-stearic acid (100 mM) was added. The suspension was incubated at 22°C for 1 min and then rapidly centrifuged at 700 × *g* for 3 min. The cellular pellet was drawn up into a 20 μl disposable capillary pipette (Corning) and one end was sealed thermally.

Pelleted plasma membranes (approx. 2.5 mg of membrane protein) were resuspended in 2 ml of 5 mM Tris-HCl, pH 7.4, and 3 μl of a methanolic solution of the doxyl-stearic acid ($5 \cdot 10^{-3}$ M) was added. The suspension was incubated for 30 min at 22°C and then 8 ml of 5 mM Tris-HCl, pH 7.4, was added. The suspension was centrifuged at 40 000 rev./min using a Spinco Type 40 rotor for 20 min and the resulting pellet was carefully drained. 5 μl of the 5 mM Tris-HCl, pH 7.4, buffer was added and the membrane carefully resuspended. The thick membrane suspension was drawn up into a 20 μl disposable capillary pipette and one end was sealed thermally.

Electron spin resonance techniques. The capillary pipette containing the sample was inserted into the rectangular TE_{120} mode cavity of an X band spectrometer and spectra were recorded at 9.05 GHz. The magnetic field strength was determined by means of an NMR magnetometer and a Hewlett-Packard 5246L frequency counter. The microwave frequency was also monitored using the same frequency counter and a Hewlett-Packard S256A plug-in unit. The power level on the sample was never greater than 10 mW. The modulation field of the 100 kHz modulation was 0.5 G. The temperature was controlled using a Varian temperature controller and was measured using a copper-constantin thermocouple inserted in the cavity. The maximum relative error in temperature is estimated to be ±0.1°C.

The spectra at each temperature were recorded with either a 5 min or a 10 min scan over 0.01 *T* (100 G) using a time constant of 0.4 s on the Ithaco 391A Lock-in amplifier. The differences between the measured spectral features for the two scan rates was well within the estimated error of ±0.3 G for measuring a peak maximum.

The rate of change of temperature was maintained at 8°C per h; usually in 2°C steps every 15 min. Thermal equilibration was carried out with the microwave bridge tuned. The following checks were carried out to ensure that there was minimal, if any, sample degradation because of the incident microwave radiation: (a) a sample left for 3 h at 20°C had identical spectra to that of a fresh sample; (b) identical results were obtained when a preparation was split and one sample was started at −10°C and taken to about 25°C and a second sample was started at 5°C and taken to about 25°C. It may also be noted that no hysteresis effects were ever observed.

Results

Lipid composition of the plasma membranes of D. discoideum

In an earlier publication it was shown that *D. discoideum* incorporated large quantities of exogenous polyunsaturated fatty acid into its cellular lipids [1].

TABLE I

THE EFFECT OF POLYUNSATURATED FATTY ACID INCORPORATION ON THE FATTY ACID COMPOSITION OF THE PLASMA MEMBRANES OF *D. DISCOIDEUM*

Percentage of total. The values, together with their standard deviations given are from three determinations. In all fatty acid abbreviations the number preceding the colon is the chain length, the number following the colon is the number of double bonds and the numbers in parentheses denote the positions of the double bonds. Thus 5,9-octadecadienoic acid is abbreviated to 18 : 2 (5,9), etc.

Fatty acid	Control	Polyunsaturated fatty acid supplemented
14 : 0	1.2 ± 0.2	1.3 ± 0.2
Palmitaldehyde	3.5 ± 0.9	2.2 ± 1.0
Palmitic acid	7.6 ± 0.5	12.2 ± 3.0
Palmitoleic acid	2.4 ± 0.2	1.1 ± 0.2
16 : 2 (5, 9), 17 : 0 *	1.3 ± 0.4	1.3 ± 0.3
18 : 0	2.7 ± 1.0	3.9 ± 0.3
18 : 1 (9) and 18 : 1 (11) *	34.6 ± 1.7	11.8 ± 1.6
18 : 2 (5,9 9) and 18 : 2 (5, 11) *	41.4 ± 0.3	3.4 ± 0.5
18 : 2 (9, 12)	—	15.7 ± 2.7
18 : 3 (5, 9, 12)	—	3.1 ± 0.6
18 : 3 (9, 12, 15)	—	11.2 ± 2.3
18 : 4 (5, 9, 12, 15)	—	3.6 ± 0.6
20 : 4 (5, 8, 11, 14)	—	19.9 ± 1.5
Others **	4.7 ± 1.8	5.6 ± 0.4
Total saturated acids	14.0 ± 0.9	18.3 ± 3.2
Total monoenoic acids	37.0 ± 1.7	12.1 ± 1.9
Total polyunsaturated fatty acid	41.4 ± 0.3	56.8 ± 2.7
Total incorporated	—	53.4 ± 6.1
Polyunsaturated fatty acid/ monounsaturated fatty acid	—	4.7
Unsaturation index	1.2	2.0

* The methyl esters of these two fatty acids are not separated under the chromatographic conditions used in this present study.

** Several unidentified minor components.

*** The sum of all fatty acids with two double bonds or more.

The data shown in Table I demonstrates that the lipids of the plasma membrane contain considerable quantities of the exogenously supplied polyunsaturated fatty acid; in fact, they account for about 50% of the fatty acid content. The degree of unsaturation in the acyl chains of the plasma membranes is clearly increased as a result of the incorporation of the exogenous polyunsaturated fatty acid. Both the unsaturation index, determined as described by Yau et al. [9] and the ratio of polyunsaturated fatty acid to monounsaturated fatty acid are increased (Table I).

TABLE II

EFFECT OF POLYUNSATURATED FATTY ACID INCORPORATION ON THE PHOSPHOLIPID AND STEROL CONTENT OF THE PLASMA MEMBRANES OF *D. DISCOIDEUM*

The values given are the means for five different preparations. The numbers in brackets are the standard deviations.

	Control	Fatty acid grown
Phospholipid (μg/mg protein)	434.8 (46.4)	425.5 (21.4)
Sterol (μg/ml protein)	99.9 (11.4)	109.4 (21.0)

In contrast the phospholipid and sterol contents of the two types of membrane are very similar (Table II). The slight increase in the sterol contents may possibly reflect some compensation for the high levels of polyunsaturated fatty acids that are incorporated. However since there is a large variation in the determined sterol levels from sample to sample, it is not clear whether this slight increase is significant.

ESR studies on whole cells

One approach that has been used to evaluate the fluidity of the surface membranes of mammalian cells is to incorporate a doxyl-fatty acid spin probe into whole cells and measure the resulting ESR spectra [10,11]. The implicit assumption in these determinations is that the relatively hydrophobic spin probe does not penetrate to the interior of the cell and, therefore, only measures the fluidity of the plasma membrane.

Intact vegetative cells of *D. discoideum* incorporated doxyl-stearic acids into the membrane, as evidenced by an intense relatively immobile ESR spectra associated with the cells. The virtual absence of sharp lines suggested that very little spin probe was present in the cytoplasm. However, the spectra rapidly disappeared and was undetectable within 30 min, suggesting either that the spin probe was reduced by surface membrane enzyme(s) or that the spin probe was internalized and then reduced. We understand that a similar rapid reduction of doxyl-stearic acid spin probes by whole cells of *D. discoideum* has been observed by other workers (Ohnishi, S., personal communication). In contrast, however, Von Dreele and Williams [17] used spin-labelled fatty acids to measure membrane fluidity of intact *D. discoideum* cells, but reported no degradation of the spin probe. The reasons for these differences are at the present time not clear.

ESR studies with isolated plasma membranes

The spectra of 5-, 12- and 16-doxyl-stearic acid incorporated into isolated plasma membranes of the cells grown in the absence of polyunsaturated fatty acids (sample A) are shown in Figs. 1, 2 and 3. The spectra reveal the well

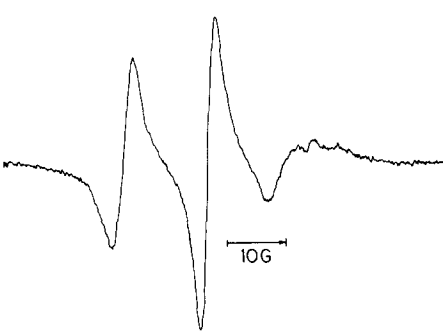

Fig. 1. The ESR spectrum of 16-doxyl-stearic acid in the plasma membranes of *D. discoideum* (control at 20° C.

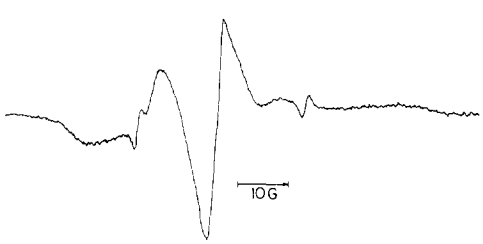

Fig. 2. The ESR spectrum of 12-doxyl-stearic acid in the plasma membranes of *D. discoideum* (control) at 20° C.

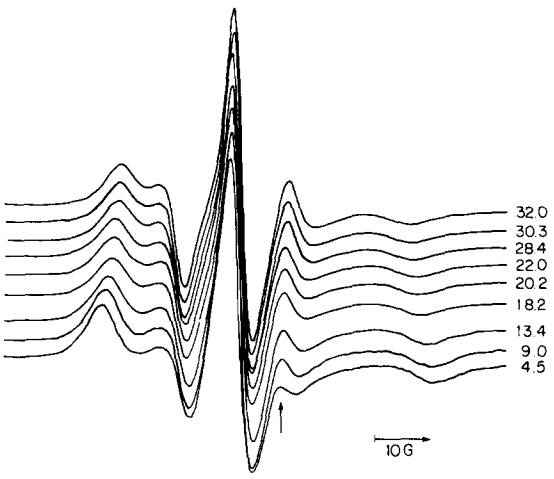

Fig. 3. The ESR spectra of 5-doxyl-stearic acid in the plasma membranes of *D. discoideum* (control) at various temperatures.

characterized gradation of motional characteristics [12] suggesting that the nitroxide probes are sampling different regions of the bilayer, indicating that the acyl chains of the spin probes are inserted at right angles to the plane of the membrane.

The ready obtention and apparent simplicity of ESR spectra belies the difficulty involved in the interpretation of the spectral lineshape. This problem has been recently stressed in an experimental study [13] and thoroughly discussed by Freed [14]. The correct interpretation of the ESR spectra of doxyl-stearic acids (or esters) requires that the system under investigation should be in the correct motional regime. Thus for example the method whereby the correlation time of the spin probe is obtained from the relative line widths of the three line nitroxide spectrum [15] requires the motion to be rapid and isotropic. Since the environment of a spin probe in a phospholipid vesicle is far from isotropic and the motion can be quite slow below about 15°C, this method of analysis for spin probes such as 16-doxyl-stearic acid (see Fig. 1) will often lead to erroneous results [13].

The alternative method of measuring the order parameter S requires that the spectra features necessary for the measurement of $2T'_{\parallel}$ and $2T'_{\perp}$ (twice the apparent hyperfine couplings of the nitroxide free radical) be clearly discernable (see for example ref. 12). Consequently, in the absence of computer simulation, the best probe to use is the 5-doxyl-stearic acid which exhibits the most ordered spectrum (compare Figs. 1, 2 and 3).

The order parameter S is given by the following expression:

$$S = (T'_{\parallel} - T'_{\perp})/[T_{zz} - \tfrac{1}{2}(T_{xx} + T_{yy})]$$

where T_{xx}, T_{yy}, and T_{zz} are the anisotropic hyperfine components of the nitroxide free radical: thus if the feature required to measure $2T'_{\perp}$ (vida infra) is not apparent at all temperatures as is often the case for biological membranes, the evaluation of S is rendered difficult, if not inaccurate. An alternative approach is to monitor the variation of $2T'_{\parallel}$ (the separation between the outer

extrema) which is linearly related to the order parameter

$$2T'_{\parallel} = 2A_0 + (4S/3)[T_{zz} + \tfrac{1}{2}(T_{xx} + T_{yy})]$$

where A_0 is the isotropic hyperfine coupling constant of the nitroxide free radical. One could, of course, evaluate S from this equation but this requires the complicating feature of including polarity effects [12] and is unnecessary in a comparative study such as this one.

The interpretation of the order parameter and hence the variation of $2T'_{\parallel}$ is not straightforward. Strictly speaking S is a function of both spatial and motional order. However, in the case of a system of randomly oriented membrane vesicles, S (and hence $2T'_{\parallel}$) can be viewed as a measure of motional order [12]. The change of $2T'_{\parallel}$ with temperature can be used to evaluate the variation of membrane fluidity with temperature, and the comparative characteristics of different membrane samples. This is the approach that has been used in the present study.

The ESR spectra of the 5-doxyl-stearic acid is shown at various temperatures for plasma membranes of cells grown in the absence of polyunsaturated fatty acid (Fig. 3). An indication of the relative mobility of the spin probe can be inferred from the temperature dependence of the feature indicated by an arrow in Fig. 3. This feature is expected to cross the base line if the motion of the probe is sufficiently rapid [14]; it is this feature that is needed to accurately measure $2T'_{\perp}$. The feature indicated in Fig. 3 becomes more prominent as the the temperature is raised and is fully apparent at 18°C for the control sample (sample A) of the plasma membranes of *D. discoideum*.

The spectra of the spin probe in membranes that had incorporated high levels of polyunsaturated fatty acid were almost identical to those in Fig. 3. The spectral feature referred to earlier, crosses the base line at 18°C, thus suggesting that the fluidity of the plasma membrane is essentially unchanged by incorporating large quantities of polyunsaturated fatty acids. The result of incorporating large quantities of polyunsaturated fatty acids has, however, a substantial affect on the stability of the spin probe in the isolated plasma membrane. Below about 25°C the spin probe appears to be relatively stable but as soon as the growth temperature is reached the spin probe is rapidly degraded; the addition of potassium ferricyanide as an anti-reductant did not prevent the loss in spin label signal. As a consequence of the spin probe degradation the temperature range employed in the study of the plasma membranes from cells which have incorporated large quantities of polyunsaturated fatty acids is from 0 to 25°C only compared to 0 to 40°C for the control. The studies of $2T'_{\parallel}$ as a function of temperature were performed on at least four samples of plasma membrane obtained from different batches of the control and polyunsaturated fatty acid-incorporated cells.

The variation $2T'_{\parallel}$ with temperature for the control and polyunsaturated fatty acid-incorporated membranes are shown in Figs. 4 and 5, respectively. There are no discontinuities in the $2T'_{\parallel}$ vs. T curves for either sample, suggesting no measurable sharp phase transition between the temperatures of observation. In Fig. 4 the dashed line is the least squares fit for the data from Fig. 5 and vice versa; this has been done since the data are so similar that whatever small differences that do exist would be obscured in a single diagram. A con-

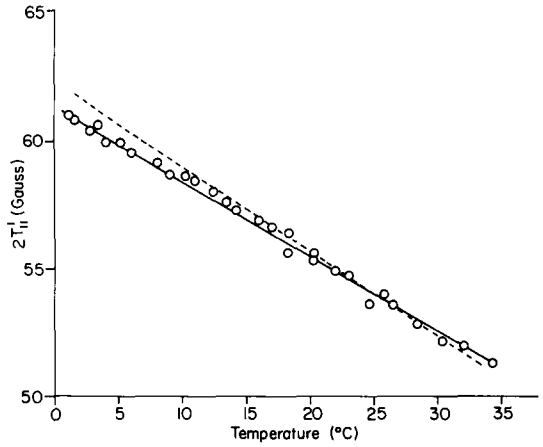

Fig. 4. The temperature variation of $2T'_{\parallel}$ for 5-doxyl-stearic acid in the control sample. The solid line represents the least squares line from 71 points. The regression parameters are: $a = -0.293 \pm 0.003$, $b = 61.3 \pm 0.1$, $r = 0.994$. The dashed line is the corresponding line for the polyunsaturated fatty acid incorporated (see Fig. 5).

sideration of Figs. 4 and 5 shows that in fact below about 20°C the fluidity, as indicated by $2T'_{\parallel}$, is slightly greater in the control and at about 25°C the fluidity is approximately equal. In fact, the results for the two samples are so similar that to all intents and purposes the fluidities are identical. The one interesting small differences is that the slope in Fig. 5 is slightly greater than in Fig. 4, suggesting that the polyunsaturated fatty acid-incorporated membrane is slightly more sensitive to temperature change. The significance of this result is not immediately apparent. These results indicate that the incorporation of polyunsaturated fatty acids has not brought about the change in fluidity that might be expected simply on the basis of an increase in the degree of unsaturation of the phospholipid.

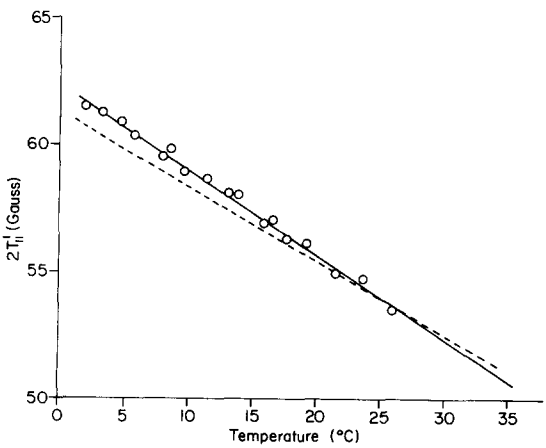

Fig. 5. The temperature variation of $2T'_{\parallel}$ for 5-doxyl-stearic acid in the polyunsaturated fatty acid-incorporated sample. The solid line represents the least squares line for 25 points. The regression parameters are: $a = -0.33 \pm 0.01$, $b = 62.3 \pm 0.2$, $r = 0.98$. The dashed line is the corresponding line for the control (see Fig. 4).

Discussion

In order to study the fluidity of the plasma membranes, we have utilized doxyl-stearic acids as spin probes. The mobility of the spin labels increased markedly with increasing distance of the nitroxide group from the carboxylate group (Figs. 1 and 2) indicating that the hydrocarbon chains of the fatty acids were inserted at right angles to the plane of the membrane, as has been found for other biological membranes [12]. We have studied in detail only the spectra of the 5-doxyl-stearic acid, since the nitroxide group of this molecule is sufficiently immobile to allow accurate determination of $2T'_{\parallel}$ which is linearly related to the order parameter.

It was shown earlier that cells containing high levels of polyunsaturated fatty acids grew normally but exhibited impaired differentiation, and it was postulated that this impaired differentiation was due to increased fluidity of the plasma membrane lipids [1]. It is possible, for example, that the conformation of contact specific proteins might be dependent upon the correct lateral compressibility of the lipid bilayer. Altering this lateral compressibility by the incorporation of lipids with highly unsaturated acyl chains into the bilayer might disrupt the active coformation of the protein. The plasma membranes of these cells do contain high levels of polyunsaturated fatty acids (Table I), but they do not exhibit an increased membrane fluidity, as determined by ESR (Figs. 4 and 5). This result has been confirmed by fluorescence depolarization measurements using diphenyl hexatriene as a probe (Tatischeff, I. and Weeks, G., unpublished). Thus the increase in the unsaturation of the acyl chains must be compensated for by other changes in membrane components. There is no evidence for an alteration in the phospholipid/protein ratio; there is a slight increase in the saturated fatty acid and sterol contents of the plasma membranes of the cells grown in the presence of polyunsaturated fatty acids, but it is not clear at the present time whether these changes are sufficient to compensate for the increase in acyl chain unsaturation. It is possible that the polar head groups of the plasma membranes phospholipids are modified and we are currently testing this possibility.

The reason for the impaired differentiation of cells grown in the presence of polyunsaturated fatty acids remains to be elucidated. It is possible that the lipids containing the polyunsaturated acyl chains binds directly to the protein components involved in cell-cell interaction, preventing their normal function. Alternatively, it is possible that cell contact is dependent upon the association of identical or non-identical contact specific molecules within the plane of each membrane. This idea stems from the evidence that concanavalin A receptor clustering is necessary for concanavalin A-mediated agglutionation [16]. It is possible that specific protein association witbin the plane of the membrane is dependent upon the presence of the correct lipid domains. Increasing the content of polyunsaturated fatty acid in a bilayer could drastically alter the nature of such domains and prevent the correct protein-protein interaction in the plane of the membrane. The ESR technique is probably insufficiently sensitive to indicate the presence of such domains.

At the present time, it is difficult to distinguish between these and other models. Furthermore, these models are not necessarily mutually exclusive.

However, it is clear from the present study that plasma membranes containing high levels of polyunsaturated fatty acids exhibit fairly normal fluidity; consequently any model postulating a gross change in fluidity leading to impaired function can be ruled out.

An interesting feature of the present study is the absence of detectable discontinuities in the plot of $T'_{\parallel}$ against temperature. In contrast in many other studies of this type [18—24], clear discontinuities in these plots have been detected and have been attributed to alterations in the state of the membrane lipid, such as the gel to liquid-crystalline phase transition or phase separation, or the onset of lipid clustering within the liquid-crystalline phase. Phase separations will usually occur in heterogeneous mixtures of phospholipids giving rise to two characteristic temperatures corresponding to the onset (t_l) and completion (t_h) of the gel to liquid-crystalline phase transition. Clustering is a manifestation of either premonitionary freezing or melting as the temperature approaches either t_h or t_l; these quasi-liquid or crystalline regions can often sequester the spin probe and cause additional discontinuities in the change of spectral parameters (e.g. S, $2T'_{\parallel}$, etc.).

All plasma membranes contain high levels of sterol, *D. discoideum* being quite typical in this regard, and the absence of detectable characteristic temperatures is, therefore, not unexpected since it has been clearly established from studies with artificial phospholipid membranes that sterols considerably broaden phospholipid phase transitions [25]. Furthermore, there are no detectable characteristic temperature transitions in the ESR spectra of the surface membrane of macrophages [26] and no temperature-dependent particle aggregation or segregation into domains in the plasma membranes of either lymphocytes [27] or *Tetrahymena* [28].

However, in some cases the presence of sterol does not abolish a detectable characteristic temperature suggesting that its effect in some membranes may be quantitatively less pronounced. For example, characteristic temperatures have been detected by ESR in the plasma membranes of cultured fibroblasts [29] and brain tissue [24] and by fluorescence in the plasma membranes of cultured fibroblasts [30].

A number of surface membrane-related phenomena, such as lectin binding and agglutination [32,33], amino acid uptake [29], and antigen movement within the plane of the membrane [34], have been shown to exhibit sharp temperature transitions which are possibly associated with phase separations of the membrane lipids. It should be noted however that breaks in Arrhenius plots of enzyme activity need not be due to lateral phase separations in the bulk lipid but rather to specific cooperative interactions between the enzyme and its phospholipid annulus [35]. Rottem et al. [31] have shown that the membranes of a variant strain of *Mycoplasma* adapted to grow with very low concentrations of cholesterol undergo a phase transition whereas none could be detected for the native strain grown in elevated levels of cholesterol. Furthermore, the ATPase activity of the variant was shown to exhibit breaks in Arrhenius plots corresponding to the phase transition, but no breaks were found in the native strain [31].

The absence of discontinuities in the temperatures variation of $2T'_{\parallel}$ no doubt reflects the presence of sterol which broadens the gel to liquid-crystalline

transition. It is also possible, however, that phase transitions do occur in the *D. discoideum* plasma membrane, but remain undetected by the 5-doxyl-stearic spin probe. Spin probes of this type do not necessarily sample the total bilayer lipid of a biological membrane [36] and there is evidence from studies on artificial membranes [37,38] that spin labels are preferentially incorporated into the most fluid regions of the lipid bilayer. It is possible that transitions in *D. discoideum* plasma membranes might be detected using a different type of spin probe, such as TEMPO or doxyl-alkanes, although in a recent study on the lipids of *Escherichia coli*, 5-doxyl-stearic acid and TEMPO detected identical characteristic temperatures [39]. Preliminary studies using TEMPO as a spin probe in the isolated plasma membranes of *D. discoideum* have shown that no discontinuities are observed in the temperature range 0—40°C (Herring, F.G., Weeks, G. and Yip, F., unpublished). In addition fluorescence depolarization studies on the plasma membranes of *D. discoideum* fail to reveal a temperature discontinuity in the temperature range 0—40°C (Tatischeff, I. and Weeks, G., unpublished) further confirming the results presented in this paper. If gel to liquid-crystalline pase separations do occur in *D. discoideum* plasma membranes, they would appear to be far less dramatic than those in most other membranes.

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