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THE FLUIDITY OF PLASMA MEMBRANES OF *DICTYOSTELIUM DISCOIDEUM*

THE EFFECTS OF POLYUNSATURATED FATTY ACID INCORPORATION ASSESSED BY FLUORESCENCE DEPOLARIZATION AND ELECTRON PARAMAGNETIC RESONANCE

F.G. HERRING [a,**], I. TATISCHEFF [a,b,*] and G. WEEKS [b]

Departments of [a] Chemistry and [b] Microbiology, University of British Columbia, Vancouver, BC V6T 1Y6 (Canada)

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Summary

Two probe techniques, fluorescence depolarization (using diphenylhexatriene) and electron paramagnetic resonance (using 5-doxyl stearic acid), have been used to assess the fluidity of the purified plasma membranes of *Dictyostelium discoideum*. Both techniques indicate that a large incorporation of polyunsaturated fatty acids into the plasma membranes does not significantly change membrane fluidity. In addition, phosphatidylcholines isolated from cells grown on both polyunsaturated fatty acid-supplemented and unsupplemented media exhibit similar mobilities of an incorporated spin probe when dispersed in aqueous solution. This result suggests that the enrichment of a membrane already high in content of fatty acyl chains containing two double bonds with those containing three or more double bonds does not markedly change fluidity.

Introduction

Modification of membrane fatty acid composition and, hence, of membrane fluidity can have marked effects on certain membrane functions. The effects of acyl chain modification on membrane enzyme activity [1] and membrane

*On leave of absence from Laboratoire Curie, 11, Rue P. et M. Curie, 75231 Paris, Cedex 05, France.
**To whom correspondence should be addressed.

transport [2] are well documented, and in addition, studies on cell adhesion [3—5], lectin-mediated aggultination [4—6] and cell fusion [9] suggest that cell-cell interactions may be profoundly influenced by membrane lipid composition.

In a previous report from this laboratory [10], it was shown that polyunsaturated fatty acid could be incorporated into the lipid of the cellular slime mould, *Dictyostelium discoideum*. Growth was unaffected by this treatment, but the subsequent differentiation of the organism was impaired. Since cell contact is essential for differentiation but not necessary for growth, it was postulated that the impaired differentiation was due to increased fluidity of the plasma membrane [10].

An electron paramagnetic resonance (EPR) analysis of highly purified plasma membrane preparations of *D. discoideum* failed to detect any alteration in the fluidity of the supplemented cells despite the incorporation of large amounts of polyunsaturated fatty acids [11]. However, in studies of animal cell plasma membranes, relatively low incorporations of polyunsaturated fatty acids have produced changes in fluidity as assessed by EPR [12].

We have therefore further examined the plasma membranes of polyunsaturated fatty acid-supplemented cells for possible changes in fluidity using fluorescence depolarization. Since probe molecules can potentially disrupt the membrane environment, the absolute reliability of results obtained by techniques that require probes must be questioned, and in this report we describe a direct comparison between EPR and fluorescence depolarization, techniques that utilize quite different probes, on the same membrane sample. The results support our original findings and show that EPR and fluorescence depolarization give qualitatively identical results.

Materials and Methods

Organism and culture conditions. Dictyostelium discoideum, strain Ax-2, was grown in a rich nutrient axenic medium supplemented with bovine serum albumin [9,11]. The polyunsaturated fatty acid supplement consisted of linoleic acid, linolenic acid and arachidonic acid, each at a final concentration of 100 mM [11,13]. Cells were grown to a density of approx. $5 \cdot 10^6$ cells/ml and then harvested by centrifugation at $700 \times g$ for 2 min. Cell pellets were washed twice by resuspension in distilled water and recentrifuged.

Membrane isolation. Washed cells were resuspended in 8.6% (w/v) sucrose, 5 mM Tris-HCl (pH 7.5). Purified plasma membranes were prepared by isopycnic sucrose density gradient centrifugation as described previously [14,15].

Fluorescence depolarization studies. All measurements were made with an instrument built by Dr. R.R. Parsons (Department of Physics, University of British Columbia). The original features of this device are as follows: the use of monochrometers instead of band filters; electronic compensation of any residual polarization; an ability to compute the polarization ($P = I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$), the anisotropy of polarization ($r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$) and the total fluorescence intensity ($F = (I_{\parallel} + 2I_{\perp})$), where $I_{\parallel}$ and $I_{\perp}$ are the components of the fluorescence signal oriented either parallel or perpendicular, respectively, to the direction of polarization of the excitiation light. Full details of this experi-

mental design are described elsewhere (Tatischeff, I. and Parsons, R.R., unpublished results).

The use of 1,6-diphenyl-1,3,5-hexatriene as a fluorescence probe in the study of liposomes and biological membranes is well established [16—20]. It has excellent spectral properties [17] and partitions equally between gel and liquid-crystalline phases [21]. The polarization, P, is related to the rotational relaxation time, $\bar{\rho}$, of diphenylhexatriene by the equation [21,22]:

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_o} - \frac{1}{3}\right) \cdot \left(1 + \frac{3\tau}{\bar{\rho}}\right)$$

where τ is the lifetime of the exited state and P_o is the polarization in the absence of rotational diffusion. Shinitzky et al. [16] have shown that this equation is applicable to non-spherical fluorophores when the absorption and emission oscillators are parallel; as they are in diphenylhexatriene. In this case, $\bar{\rho}$ represents the average of rotational relaxation times for in- and out-of-plane reorientation. The direct measurement of $\bar{\rho}$ is desirable for a more precise assessment of membrane fluidity [19]. Such measurements require the direct measurement of τ [19], a typical value of τ for diphenylhexatriene is 9.5 ns. In the absence of measurements of τ, the value of P will be an indicator of membrane fluidity provided that τ does not change dramatically from sample to sample. The variation of τ with lipid composition has been studied by Gilmore et al. [19] who showed that the variation of τ was only 0.5 ns for a wide variety of lipid mixtures. Consequently, the determination of P as a function of membrane composition should reflect changes in the rotational relaxation rate and hence changes in structural organization, i.e., fluidity. In fact, P will be related to membrane order and viscosity in much the same way that $2T'_{\parallel}$, as determined in EPR measurements [11], is related to these membrane properties.

Determination of phospholipid composition. Lipid was extracted from plasma membrane preparations by using the method of Bligh and Dyer [23]. The extracted lipid was resolved into its constituent phospholipids by two-dimensional thin-layer chromatography [24] and the individual phospholipid constituents were determined by using the method of Barlett [25].

Fluorescence depolarization studies. Incorporation of the diphenylhexatriene was accomplished by incubation of equal volumes of purified plasma membranes (0.4 mg/ml) or intact cells ($7.6 \cdot 10^6$ cells/ml), suspended in 10 mM Tris-HCl (pH 7.4 solution), and diphenylhexatriene, $4 \cdot 10^{-6}$ M in deionized water. The diphenylhexatriene solution was prepared by vortex mixing 1 part of $2 \cdot 10^{-3}$ M diphenylhexatriene in tetrahydrofuran with 500 parts of deionized water. The diphenylhexatriene incorporation into the membranes was followed by the fluorescence intensity as a function of time, inasmuch as the probe is devoid of any fluorescence in an aqueous environment. Unless otherwise indicated, measurements were made at 20°C. Since the temperature of the heating block was measured rather than that of the cuvette contents, there may be a systematic error in the temperature measurements, but this does not influence any of the conclusions reached in this study.

Phosphatidylcholine preparation and EPR experiments. Lipid was extracted

from *D. discoideum* cells by using the method of Bligh and Dyer [23]. The lipid extracts were dried under N_2 and extracted with acetone/Solid CO_2 to remove neutral lipid. The remaining lipid was chromatographed on silica gel thin-layer plates using $CHCl_3/CH_3OH/H_2O$ (70 : 30 : 4, v/v) developing solvent. The resulting phosphatidylcholine fraction was eluted from the plates by $CHCl_3/CH_3OH$/(2 : 1, v/v) and was rechromatographed employing the same thin-layer chromatographic procedure. The purified phosphatidylcholine was checked for purity by two-dimensional thin-layer chromatography [24] and was found to contain less than 1% contaminating phospholipid.

Results and Discussion

Fluorescence depolarization studies on plasma membranes

In a previous EPR study, it was shown that the fluidity of the plasma membrane of *D. discoideum* did not increase when large quantities of polyunsaturated fatty acids (53% of the total fatty acid) were incorporated [11]. In view of the somewhat surprising nature of the result, we decided to utilize a second, independent physical method to compare the fluidities of polyunsaturated fatty acid-supplemented and unsupplemented plasma membranes and chose the fluorescence depolarization technique using diphenylhexatriene as probe [16–18].

Fig. 1a shows the time course of the incorporation of diphenylhexatriene into the two different plasma membrane preparations. There was little further increase in fluorescence intensity after 20 min, suggesting that the incorporation of the probe was complete as this time. The polyunsaturated fatty acid-supplemented membrane was saturated earlier and did not attain as high a fluorescence intensity as the control membrane (Fig. 1), but the reason for this difference is not understood at present. The fluorescence depolarization ratios for both samples remain constant and are probably measures of the average

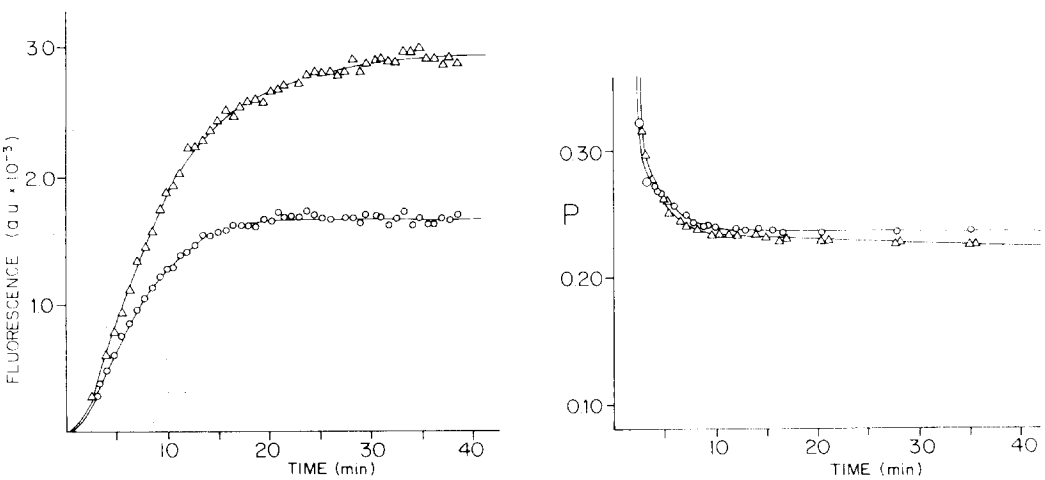

Fig. 1. (a) Total fluorescence intensity and (b) fluorescence depolarization as a function of time following the incorporation of diphenylhexatriene into unsupplemented (○) and polyunsaturated fatty acid-supplemented (△) plasma membranes of *D. discoideum*. a.u., arbitrary units.

TABLE I

COMPARISON OF THE FLUORESCENCE DEPOLARIZATION (FD) AND EPR TECHNIQUES (22°C)

Estimated errors for EPR, ±0.3; estimated errors for FD, ±0.003. PUFA, polyunsaturated fatty acids.

Plasma membrane preparation	EPR $2T'_{\parallel}$ (G)	FD $\overline{P}$
Unsupplemented	54.5	0.228
Unsupplemented	54.3	0.220
PUFA supplemented	54.6	0.235

fluidity of all environments that the probe samples. The fluorescence polarization measurements for the two samples were almost equal (Table I), suggesting that the fluidities of the two membranes were the same.

Table I shows a direct comparison between the fluorescence depolarization results and EPR measurements made on the same sample of membranes. The EPR results are identical within experimental error to those previously published [11]. Clearly, neither technique demonstrates a marked increase in membrane fluidity in the membranes from polyunsaturated fatty acid-supplemented cells.

Fig. 2 shows the influence of temperature on the polarization ratio of diphenylhexatriene in the two samples of plasma membrane. There is only a slight difference in the two slopes, indicating that polyunsaturated fatty acid supplementation does not produce a pronounced change in fluidity at any temperature. In addition, there is no discontinuity in the plots of polarization ratio vs. temperature, suggesting that there is no detectable phase transition in *D. discoideum* plasma membranes, a result in accord with that previously obtained using EPR [11].

One interesting feature in Fig. 2 is that the two lines intersect at approx. 22°C as did the temperature profiles of $2T'_{\parallel}$ in the earlier EPR study [11].

Thus, the fluorescence depolarization technique gives results that are qualitatively similar to those obtained by EPR. The exact location of the diphenyl-

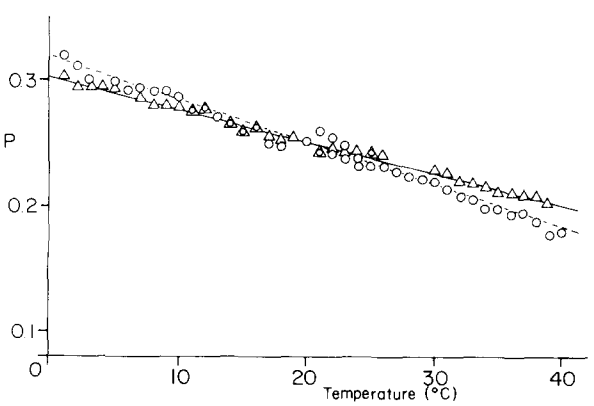

Fig. 2. The effect of temperature on the fluorescence depolarization of diphenylhexatriene in unsupplemented (○) and polyunsaturated fatty acid-supplemented (△) plasma membranes.

hexatriene in the bilayer is not known, but it is likely that it probes a region deeper than the 5-doxyl stearic acid probe. An important question is whether the motion of a probe molecule directly measures its environment or merely reflects the way in which it perturbs its environment, since all probes will perturb the membrane environment to some degree. Thus, if two distinct probes (5-doxyl stearate and diphenylhexatriene) monitoring different regions of the bilayer, displaying different physical properties and having quite different structural features, give identical qualitative measurements of fluidity, then it is likely that both probes are reflecting their environment rather than changes in a perturbed environment. Consequently, we feel that the comparative study carried out here confirms, firstly, that there is no phase transition in the plasma membranes of *D. discoideum* and, secondly, that incorporation of large quantities of polyunsaturated fatty acids into these plasma membranes does not alter membrane fluidity.

Phospholipid composition of polyunsaturated fatty acid-supplemented and unsupplemented plasma membranes

It is clear from the discussion in the foregoing section that the fluidity of the plasma membrane of *D. discoideum* is unaffected by the incorporation of large quantities of polyunsaturated fatty acids. It has been shown recently, however, by King and Spector [12] that the incorporation of relatively small quantities of polyunsaturated fatty acids increases the membrane fluidity of Ehrlich ascites cell plasma membranes. Since variations in the amounts of other membrane components are known to alter membrane fluidity (e.g., changes in phospholipid:sterol ratios), it is possible that alterations in the levels of other *D. discoideum* plasma membrane constituents compensate for the polyunsaturated fatty acid incorporation. We have shown previously, however, that polyunsaturated fatty acid supplementation induces no change in the level of total phospholipid or sterol [11], and the data in Table II show that there is also no change in the relative amounts of the various individual phospholipid constituents.

TABLE II

PHOSPHOLIPID COMPOSITION OF PURIFIED PLASMA MEMBRANES OF *D. DISCOIDEUM*

The values given are the means for three determinations ± S.E. Results are expressed as percent total phospholipid.

Phospholipid	Unsupplemented	Supplemented
Phosphatidylcholine	28.8 ± 2.2	30.0 ± 1.9
Phosphatidylethanolamine	27.8 ± 4.2	28.6 ± 1.9
Phosphatidylethanolamine (plasmalogen form)	27.3 ± 3.8	23.3 ± 4.8
Phosphatidylinositol	7.8 ± 2.0	8.8 ± 0.5
Cardiolipin	2.0 ± 0.8	1.3 ± 0.5
Phosphatidylserine	1.5 ± 0.8	1.2 ± 0.8
Lysophosphatidylcholine	1.5 ± 0.3	2.3 ± 1.2
Lysophosphatidylethanolamine	1.4 ± 0.6	2.0 ± 0.5
Phosphatidylglycerol	1.2 ± 0.1	1.3 ± 0.1
Phosphatidic acid	0.3 ± 0.3	0.5 ± 0.3
Unidentified phospholipids	1.2 ± 0.8	0.7 ± 0.4

EPR studies of phosphatidylcholine from D. discoideum

The reason for the failure of polyunsaturated fatty acids to increase membrane fluidity is puzzling, particularly in the light of the recent experiments of King and Spector [12]. However, there have been very few studies that have systematically examined the effects of polyunsaturated fatty acids on membrane fluidity. While it is well established that an alteration in the saturated-to-monounsaturated fatty acyl chain ratio profoundly affects membrane fluidity [26], the magnitude of the effect of further increases in the degree of unsaturation are not known. It is conceivable that the latter effects are far less dramatic.

In order to investigate this question more thoroughly, we have purified phosphatidylcholine from unsupplemented and polyunsaturated fatty acid-supplemented cells and measured $2T'_{\parallel}$ of 5-doxyl stearic acid as an indicator of the effect of polyunsaturated fatty acids on the fluidity of dispersions of a single phospholipid constituent.

The fatty acid compositions of the purified phosphatidylcholines are shown in Table III. The difference between the control and polyunsaturated fatty acid-enriched samples is less pronounced in comparison to the data for the total membrane fatty acid composition [11]. Nonetheless, there is considerable replacement of dienoic acids with polyenoic acids (Table III) in the phosphatidylcholine moiety. This change in fatty acid unsaturation produces only a small effect on membrane fluidity as measured by EPR (Fig. 3). The small

TABLE III

FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE

Each value is the mean of two determinations ± S.E. Results are expressed as % total fatty acid. PUFA, polyunsaturated fatty acids. 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 number following Δ denotes the position of the double bonds.

Fatty acid	Unsupplemented	PUFA supplemented
14:0	1.0 ± 1.0	0.2 ± 0.2
Palmitaldehyde	0	0
16:0	9.2 ± 1.6	12.2 ± 5.1
16:1 (Δ^9)	3.2 ± 0.3	2.8 ± 0.1
16:2 ($\Delta^{5,9}$) and 17:0 [a]	3.3 ± 0.2	0.5 ± 0.5
18:0	3.8 ± 0.6	4.7 ± 1.0
18:1 (Δ^9 and Δ^{11}) a	17.8 ± 1.3	15.9 ± 0.1
18:2 ($\Delta^{5,9}$ and $\Delta^{5,11}$) a	60.4 ± 2.5	20.8 ± 1.6
18:2 ($\Delta^{9,12}$)	0	7.4 ± 0.4
18:3 ($\Delta^{5,9,12}$)	0	4.3 ± 0.2
18:3 ($\Delta^{9,12,15}$)	0	4.4 ± 0
18:4 ($\Delta^{5,9,12,15}$)	0	9.0 ± 0.7
20:4 ($\Delta^{5,8,11,14}$)	0	16.3 ± 0.8
Others [b]	1.3 ± 1.0	1.5 ± 0.7
Total saturated	14.0	17.1
Total monoenoic	21.0	15.9
Total dienoic [c]	60.4	28.2
Total polyenoic [d]	0	34.0

a The methyl esters of these fatty acids are not resolved by the chromatographic conditions used in the present study.

b Several unidentified minor components.

c Number does not include 16:2 ($\Delta^{5,9}$), see footnote a.

d Fatty acids containing three or more double bonds.

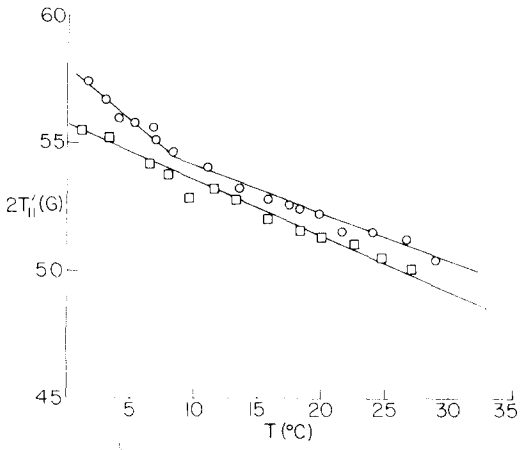

Fig. 3. The effect of temperature on $2T'$ for dispersions of 5-doxyl stearic acid and phosphatidylcholine prepared from unsupplemented (○) and polyunsaturated fatty acid-supplemented (△) cells.

difference in $2T'_{\parallel}$ in the region of the growth temperature 22°C) is statistically significant at 95% confidence level, but corresponds roughly to an order parameter change of less than 0.02. Thus, despite the marked difference in their fatty acid composition (Table III), only a small change in fluidity occurs, suggesting that the enrichment of phospholipids which already have a high proportion of fatty acyl chains containing two double bonds with those containing three and four double bonds does not cause a marked decrease in membrane fluidity.

We note here that at low temperatures, a discontinuity exists in the temperature profile of $2T'_{\parallel}$ for the unsupplemented phosphatidylcholines (Fig. 3). The significance of the discontinuity is not clear at the present time and is absent in the purified plasma membranes [11].

We have demonstrated that fluorescence depolarization and EPR provide essentially the same qualitative results wth regard to membrane fluidity, despite the fact that the probes used in both cases are markedly different in structure. The two techniques confirm the fact that the incorporation of large amounts of polyunsaturated fatty acids with three and four double bonds does not appear to dramatically change the fluidity of the *D. discoideum* plasma membrane. EPR studies on highly purified phosphatidylcholines from *D. discoideum* plasma membranes also tend to support this conclusion, although the enrichment of polyenoic fatty acids in this single species is less pronounced as compared to the total lipid. A systematic EPR or fluorescence depolarization study of chemically synthesized phosphatidylcholines with well defined fatty acid composition will be necessary to substantiate further the effects of polyenoic acids on membrane fluidity.

Note added in proof (Received August 13th, 1980)

In a recent study, Stubbs et al. (Biochemistry (1980) 19, 2756–2762) showed that although the composition of lymphocyte plasma membrane can be

modified by exogenous fatty acids with a greater degree of unsaturation, there is little effect on the degree of fluidity of the membrane as monitored by the effects on the rotational relaxation time of diphenylhexatriene.

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